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Isolation and characterization of bacterial isolates from chilli (*Capsicum annuum* L.) rhizosphere as potent plant growth promoter

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ABSTRACT

The present study was carried out at Department of Botany, University of Kalyani, Kalyani, Nadia,West Bengal, with the objective to isolate, characterize chilli rhizospheric soil bacteria and record their plant growth promoting rhizobaetana (PGPR) activity potential in southern West Bengal as a model. Chilli plants were collected from local farmers' fields. Bacterial strains were collected from rhizospheric soil, rhizoplane and root invaded condition by dilution plate count technique employing 4-different media. The isolated bacterial strains were characterized by their morphological, cultural, staining and biochemical properties. Salt and pH tolerance also been studied. Different direct plant growth promoting activities of the bacterial isolates viz. ammonia production, phosphate solubilization, siderophore production, IAA production and indirect activities such as hydrocyanic acid (HCN) production, against antagonistic effect on plant pathogenic bacteria (Pseudomonas, Xanthomonas) and fungus (Fusarium) were studied. It was found that out of 36 isolates, except 4 isolates all showed multifunctional PGPR and bio-control activity. These multifunctional PGPR may be used for improvement of crop performance in chilli and they may be formulated to be used as biofertilizers for safe farming practices.

Key words: Growth, promoter and rhizosphere.

Chilli (*Capsicum annuum* L.) is popular around the world for its colour, flavour, degree of pungency, nutritive value and as a spice – vegetable, it is of high demand due to its taste and unlimited utility all over India and the world (Tiwari *et al,* 2005). India is the biggest producer of chilli and has a great diversity and wide variability in shape, size, colour and degree of pungency. Of late, interest in soil microorganism as a diverse group of soil organisms that promote plant growth (Bashan, 1998) or help to prevent the attack of soil-borne plant pathogens has taken prominence (Chanway, 1997). Such beneficial free living soil bacteria are usually referred to as Plant Growth Promoting Rhizobacteria or PGPR (Kloepper et.al. 1989).

The benefits that the PGPR offer are: stimulation of growth promoting hormones (Gutierrez Man˜ ero *et al.*, 1996; Barazani and Friedman, 1999; Gutierrez Man˜ ero *et al.,* 2001), solubilization of phosphorus (de Freitas et al.,1997; Vazquez *et al.*, 2000), siderophore production (Kloepper et al., 1980; Raaska *et al.,* 1993), antibiotic secretion (Schnider et al., 1994), inhibition of plant ethylene production (Glick *et al.*, 1994, 1997) and induction of systemic resistance to plants against pathogens (Zehnder *et al.,* 2000; Ramamoorthy *et al.*, 2001).So, as the bacteria are in direct contact with root tissues and perform various activities, it is imperative that root colonization is essential for growth promotion (Kloepper and Beauchamp, 1992, Ikeda *et al.* 1998).The present study was performed to isolate, characterize chilli rhizospheric soil bacteria and record their PGPR activity potential in southern West Bengal as a model.

MATERIALS AND METHODS

The present study was carried out in the microbiology laboratory, Department of Botany, University of Kalyani, Kalyani, Nadia,West Bengal, India, during autumn-winter season of 2005-2006. Seedlings (cv. Suryamukhi) were collected from local farmers' fields (from Madandanga village near Kalyani, Nadia). Plants of 5 to 6 week age (after seed sowing) were uprooted and the soil samples at the vicinity of root zone (rhizosphere) were collected in clean polythin bags, brought to the laboratory and stored in aseptic condition for further use.

Isolation of bacteria

Bacterial strains from collected soil samples were isolated by soil dilution plate count technique employing 4-different media such as plate count agar), yeast extract mannitol agar, Thronton's medium and a synthetic medium. Plates were incubated at 28 ±2°C and after 48-72 ho++urs of incubation the number of bacterial colonies developed was recorded and inoculated separately in agar slopes maintained at 4°C.

Rhizoplane bacteria were isolated by cutting the root portion in pieces of 3-5 cm length in a conical flask containing 30 ml of sterile distilled water. The flasks were shaken vigorously for 30 minutes and the water samples of the flasks were serially diluted and finally plated using the 4-different media mentioned earlier.

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Root endophytes were isolated by excision of root region (3-5 cm length) and homogenized with sterile distilled water after surface sterilization of root pieces with 0.1% mercuric chloride solution (for one min) followed by washing several times with sterile distilled water. The endophytes bacteria were isolated from homogenized root mixture by plating on the media previously used.

Characterization of bacteria

The isolated bacterial strains were characterized by their morphological, cultural, staining and biochemical properties. Morphological characters include colour, elevation, edge of the colony and presence or absence of capsule in individual isolates. Gram nature of each isolates was initially determined by using crystal violet and safranin stain.

The biochemical characterization (amylase, catalase, gelatin hydrolysis, citrate utilization, H_2S production, methyl red, urease) of the isolates were performed by standard microbiological techniques.

Carbohydrate utilization test was performed by inoculating isolated bacteria in synthetic medium broth impregnated with various carbohydrates such as arabinose, cellobiose, dextrose, fructose, galactose, inositol, lactose, mannitol, moltose, raffinose and sucrose and incubated at 28±2^{0}C for 48 hours. Glucose was replaced by various other sugars based on the carbon content of glucose. After incubation the growth was measured at 600 nm.

Tolerance to salt and pH by isolated bacteria

Bacterial isolates were separately inoculated in the nutrient agar medium (beef extract 5.0 g; peptone 5.0 g; NaCl 5.0 g; glucose 1.0 g; agar 10 g; distilled water 1 lit pH 7.0±0.2) with different concentration of NaCl such as 0.5%, 5%, 10%, 20%, 30% was added to the medium for maintaining salt concentration and incubated for 48 hours at 28±2^{0}C. It was also studied that each of the bacterial isolates inoculated into the plate count agar medium of different pH (4, 6, 8, 10 and 12), which was adjusted by using of 1N NaOH and 4N HCl. After incubation, the growth of the isolates in different pH medium was recorded.

Biochemical study of the isolated bacteria for their plant growth promoting (PGP) ability and bio-control activity

Different direct plant growth promoting activities of the bacterial isolates viz. ammonia production, phosphate solubilization, siderophore production, IAA production and indirect activities such as hydrocyanic acid (HCN) production, against antagonistic effect on plant pathogenic bacteria (*Pseudomonas*, *Xanthomonas*) and fungus (*Fusarium*) were studied as depicted hereunder.

Ammonia production was determined by the method of Dye (1962). Individual bacterial isolate inoculated in peptone water broth of pH 7.0) and incubated at 28±2^{0}C for 4 days. After incubation period, 1ml Nesslers' reagent was added to the tubes. Change of colour of the broth indicates the ability of each isolate for ammonia production.

Phosphate solubilizing ability of the isolated bacteria was measured by following standard microbiological techniques, especially by Pikovskaya's medium (HIMEDIA).

Siderophore productiom by each of the isolated bacterial strain was determined by the method of Schwyn and Neilands (1987). Bacterial isolates were separately inoculated in chromazurol S (CAS) blue agar medium which is prepared by adding two solutions (solution A and solution B) as follows **Solution A**: 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution [1 mM FeCl3.6H2O, 10mM HCl] and slowly added to 72.9 mg HDTMA [hexadecyltrimethylammonium bromide] dissolved in 40 ml water. The resultant dark blue liquid was autoclaved.

Solution B: a basal medium containing defferated 1M sucrose 3ml; deferrated 1M $CaCl_2$ 0.4 ml; defferated 1M $MgSO_4.7H_2O$ 0.8 ml; defferated 2% K_2HPO_4 10 ml; NaCl 0.2 g; $NaMoO_4$ 0.005 g; Pipes [free acid] 30.24 g and agar 15 g; in 800 ml water was prepared. The pH of the medium was adjusted to 6.8 by the addition of 50% [w/w] NaOH and autoclaved. After cooling to 50^{0}C; 30 ml of defferated casamino acids [10%] was added as sterile solutions. Finally solution A was added to solution B along the glass wall with enough agitation to achieve maximum mixing without formation of foam and incubated for 24 hours at 28±2^{0}C. After incubation, yellow to orange halozone was observed around the bacterial colony.

IAA production was ascertained by the method of Gordon and Weber (1951). 1ml of each freshly cultured bacterial suspension was transferred separately into 20 ml nutrient broth in tube supplemented with L-tryptophan (0.2%) or without L-tryptophan. After 24 hours of incubation, growth Optical Density was observed ;then centrifuged at 8000g for 10 minute, 2 ml of supernatant and 2 ml of salkowski reagent (100 ml of 35% perchloric acid + 2 ml 0.5M $FeCl_3$ solution[freshly prepared]) were mixed and incubated for 30 minute in dark. After incubation colorimetric estimation of each individual isolate was done at 530 nm.

The potential use of the bacterial isolates as bio-control agents was ascertained by using virulent phytopathogenic strains of *Xanthomonas* sp., *Pseudomonas* sp. and *Fusarium* sp. The interaction was performed on agar plate in aseptic condition initially by streak methods followed by cup method in Petri plates.

HCN production was determined by the method of Bakker and Schipper (1987); the bacterial

isolates were inoculated by streak method in Petri plates containing sterile King's B (proteose peptone 20 g; glycerol 10 g; K_2HPO_4 1.50 g; $MgSO_4.7H_2O$ 1.50 g; agar 15 g; pH 7.2) medium amended with 4.4 g/lit of glycine. Then Whatman no.1 filter paper was soaked in 0.5% picric acid in 2% sodium carbonate solution and placed in the lid of Petri plate. The Petri plate was sealed with parafilm and incubated for 4 days at 28±2^{0}C. After incubation, change of color in filter paper was observed in comparison to control.

RESULTS AND DISCUSSION

In recent years, many studies have revealed that the ability of both the Gram-positive and Gram-negative bacteria to go into a viable but non-culturable (VBNC) state where bacteria are still viable, show metabolic activity and respiration but, cannot be shown as colony forming units by the conventional plate counts on standard microbiological media (Sardessai, 2005 and De Fede and Sexstone, 2001). For that reason different types of medium are used for bacteria isolation to collect a number of different types of microorganisms. A synthetic medium was also used for low nutrient capacity as like soil environment.

The observation on the colony morphology, shape and Gram nature of the isolated bacterial strains is presented in table 1. It is apparent from the table that the 36- bacterial isolates were distinctly different from each other by their colour and gross colony morphology. Initially it was noted that out of 36 isolates, 29 were whitish in colour, 5 were yellowish and the rest two were glistering. It was also observed that most of the bacterial colonies were circular in form, entire in margin, flat and opaque in nature (Table 1). It was further noted that only 30.55% of the isolates were irregular in form, 27.77% of the isolates had margins other than entire and 13.88% isolates were translucent and only 22 % of isolates were raised in elevation. The results clearly indicate that rhizospheric soil possesses more bacteria, both quantitatively and qualitatively, as compared to rhizoplane and root endophytic condition. Seldin *et. al.* (1998) reported that the microbial diversity diminished in rhizoplane from rhizosphere at the plant-soil interface. Basically the isolated bacterial cells were of 2 types, either rod or cocci. Twenty seven isolates out of 36 were rod shaped and the rest nine were cocci. Both the types of cells were obtained either singly or in chain. Further more, it was noted that out of 36 isolates, 31 strains were Gram positive and the rest 5 were Gram negative as noted in the initial typical crystal violet followed by safranin stain. The results clearly indicate that chilli rhizospheric soil environment Gram positive bacteria were predominant than Gram negative bacteria. It is also noted that 86 per cent of bacteria showed Gram positiveness and 14 per cent showed Gram negetiveness.

The isolated bacteria have further to be characterized biochemically as a routine work by a number of biochemical tests such as amylase, catalase, gelatin hydrolysis, methyl red test, H_2S production, citrate utilization, urease and their carbohydrate utilization capacity. It was revealed from the study that out of 36 isolates 21 showed positive results in amylase test whereas rest 15 showed negative results. In catalase test, 31 isolates showed positiveness whereas only 5 showed negative characters. The degree of positiveness is however, variable. Out of 31 strains that showed positiveness 18 was strongly positive as indicated by triple + sign in table 2 whereas 13 strains showed either moderate or feeble positiveness as indicated by either double or single + sign. The results further revealed that out of 36 isolates only 5 were able to hydrolyze gelatin present in the medium and 5 showed methyl red positive. It was of interest to note that the isolate C5 is the lone bacterium among the isolates which showed positiveness in both gelatin hydrolysis and methyl red test. Moreover, it was also noted that isolate C16 is the only isolate which was able to produce H_2S. The results further indicated that out of 36 only 7 isolates were able to utilize citrate and only 6 isolates were able to utilize urease. Eleven different sugars and sugar alcohols were separately incorporated in broth medium as carbon source, inoculated separately with each of the bacterial isolates and incubated. The result indicated that the ability of utilization of the different sugars by the test isolates was variable but most of the organism prefers to use simple sugar, such as dextrose or fructose. Few isolates such as C3, C6, C22, C23, and C36 however, preferred sugar alcohol such as mannitol or inositol also with various other sugars.

Microbes in alkaline soils in India are confronted with high salt, high pH, and high temperature and phosphate solubilization by microbes is highly sensitive to these environmental stresses (Johri *et al.*, 1999). It is necessary for any PGPR that they should withstand different environmental conditions like elevated pH, salt, and temperature. The capability to withstand the adverse environmental conditions such as high salinity, high/low pH, and high temperature is significant not only for rhizobacterial survival in tropical agricultural soils but also to be used as biofertilizer (Banerjee *et. al.* ,2010).The capability of growth of the isolates in medium having different concentration of salts and pH range was examined. The data in Table 3 depicts that at 0.5% salt concentration all the isolates grew well. Except few, most of the isolates adjusted to grow at 5% salt concentration too but the number was remarkably reduced at 10% concentration. Concentration of more than 10% was intolerable for all the isolates and not a

single organism could grow at concentrations more than 10%. In contrast, all the isolates had a wide range of pH tolerance. Although the very few test bacterial isolates (only 12%) were able to grow at low pH; but all the other test isolates could grow well at a pH range of 6 to 10 and about 56% of them could even withstand a pH of 12.

Table 4 shows the ability of the isolates to produce ammonia, siderophores, IAA, solubilize phosphate and as biocontrol agent. It is apparent from the results that the PGPR activities of the test isolates were variable. Out of 36 isolates only 6 were able to produce ammonia, 14 could solubilize phosphate, 12 were able to produce siderophore and 31 isolates were able to produce IAA. Individual test isolates was examined whether they are able to check the growth of plant pathogenic bacteria such as *Pseudomonas*, *Xanthomonas* and a fungus *Fusarium* on dual culture agar plate. It was noted from the results that most of the organisms were inhibitory to the growth of one, two or three of the pathogenic organisms. The results further showed that out of 36 bacterial isolates 26 were inhibitory to the growth of *Xanthromonas* and 11 were able to inhibit the growth of *Fusarium*. The growth of *Pseudomonas* was not much affected by the test isolates and only 4 of the isolates gave positive response in this regard. It is found that 13.88% bacterial isolates are able to produce HCN. In many cases a single PGPR displays several modes of action. Similarly, the isolation and characterization of bacteria from rhizosphere often reveals their ability of multiple modes of action from the population of putative PGPR inhabiting the rhizosphere. Antoun *et al* (1998) recorded similar results; they surveyed 266 strains of rhizobia and found 83% produced siderophores, 58% produced IAA, and 54% could solubilize phosphorus; they also noted that when inoculated radish with these strains , it revealed 25% of the strains to be PGPR, 64% producing no effect, and 11% having detrimental effects on plant growth. Husen (2003) reported that out of 14 rhizospheric soil bacteria isolated from Bohol and Tarlac, Philippines, 12 showed IAA producers, 7 showed positive responses in siderophore production and 4 isolate showed phosphate solubilizer. Upadhyay and Shrivastava (2010) reported that *Pseudomonas fluorescens* strain Psd showed multifunctional plant growth promoting property.

These multifunctional PGPR may be used for improvement of crop performance in chilli and other crops and they may be formulated to be used as biofertilizers.

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Table 1: Preliminary characterization of bacteria isolated from chilli rhizospheric soil, rhizoplane and root invading condition

Source of isolate	Isolates	Colony characters					Shape and Gram nature of bacteria	
		Colour	Form	Margin	Elevation	Density	Shape[a]	Gram Nature[b]
Rhizospheric soil (20) *	C1	Yellow	Circular	Lobate	Flat	Opaque	Rod, single cell	[c]+ ve
	C2	White	Irregular	Erose	Raised	Opaque	Rod , cells are in chain	+ ve
	C3	Yellow	Circular	Undulate	Flat	Opaque	Rod , cells are in chain	+ ve
	C4	White	Irregular	Erose	Flat	Opaque	Rod, single cell	+ ve
	C5	White	Circular	Entire	Flat	Opaque	Rod , cells are in chain	+ ve
	C6	White	Irregular	Lobate	Flat	Opaque	Rod, cells small in size	+ ve
	C7	Yellow	Circular	Entire	Flat	Opaque	Rod , cells are in chain	+ ve
	C8	White	Rhizoidal	Filamentous	Flat	Opaque	Rod, cells are in long chain	+ ve
	C9	White	Irregular	Lobate	Raised	Opaque	Rod, single cell	+ ve
	C10	White	Circular	Entire	Convex	Opaque	Rod, single cell	+ ve
	C11	White	Circular	Erose	Raised	Opaque	Rod , cells are in chain	+ ve
	C12	Yellow	Circular	Entire	Raised	Opaque	Rod , spore forming	+ ve
	C13	White	Irregular	Undulate	Flat	Opaque	Rod , cells are in chain	+ ve
	C14	White	Irregular	Lobate	Convex	Opaque	Rod, single cell	+ ve
	C15	White	Circular	Curled	Flat	Opaque	Rod , cells are in chain	+ ve
	C16	White	Circular	Undulate	Flat	Opaque	Rod , cells are in chain	+ ve
	C17	White	Circular	Curled	Raised	Opaque	Rod, small in Size	+ ve
	C18	White	Irregular	Undulate	Convex	Opaque	Rod, thick wall, small in size	+ ve
	C19	White	Irregular	Lobate	Flat	Translucent	Rod , small spore forming	+ ve
	C20	White	Irregular	Filamentous	Flat	Opaque	Rod, some are in chain	+ ve
Rhizoplane (9) *	C21	White	Circular	Curled	convex	Opaque	Coccus, cells are in chain	+ ve
	C22	White	Circular	Filamentous	Flat	Opaque	Coccus, single cell	+ ve
	C23	White	Circular	Erose	Convex	Opaque	Rod , cells are in chain	- ve
	C24	White	Circular	Entire	Flat	Translucent	Oval, cells are in chain	+ ve
	C25	Greyish-white	Circular	Entire	Pulvinate	Opaque	Oval to rod, cells are in chain	+ ve
	C26	White	Spindle	Entire	Flat	Opaque	Rod , cells are in chain	+ ve
	C27	White	Circular	Undulate	Raised	Opaque	Coccus, single cell	- ve
	C28	White	Irregular	Filamentous	Flat	Opaque	Coccus, single cell	+ ve
	C29	Glistering	Circular	Entire	Flat	Opaque	Coccus, single cell	+ ve
Within the Root (7) *	C30	White	Spindle	Entire	Raised	Opaque	Rod , spore forming	- ve
	C31	Glistering	Circular	Entire	Flat	Translucent	Rod , spore forming	- ve
	C32	White	Irregular	Curled	Flat	Opaque	Rod, single cell	+ ve
	C33	White	Circular	Entire	Raised	Translucent	Rod , cells are in chain	+ ve
	C34	White	Circular	Erose	Pulvinate	Opaque	Rod, single cell	+ ve
	C35	Orangish-yellow	Circular	Entire	Flat	Opaque	Rod, single cell	+ ve
	C36	White	Circular	Undulate	Flat	Translucent	Coccus, cells are in chain	- ve

** Figure in the parentheses indicate the total number of bacteria obtained in a particular habit, [a] Shape of the individual cell was seen under microscope (10 x 100), [b] Gram nature of the individual bacterial isolate was determined by typical Gram staining with crystal violet followed by saffranin, [c]"+" or "-" sign show the positive or negative reaction*

Table 2: Effect of various salt concentrations and pH on the growth of bacterial isolates

Sl. No.	Isolates	salt concentration[a]					pH values[b]				
		0.5 %	5%	10 %	20 %	30 %	4	6	8	10	12
1	C1	+++[c]	+++	-	-	-	-	++	++	++	++
2	C2	+++	+++	++	-	-	++	++	++	++	-
3	C3	+++	++	-	-	-	-	++	++	++	++
4	C4	+++	+++	+	-	-	+	++	++	++	-
5	C5	+++	++	-	-	-	+	++	++	++	++
6	C6	+++	++	++	-	-	-	++	++	++	+
7	C7	+++	+++	-	-	-	-	++	++	++	++
8	C8	+++	-	-	-	-	-	++	++	++	-
9	C9	+++	+++	+	-	-	+	++	++	++	+
10	C10	+++	++	-	-	-	-	++	++	++	++
11	C11	+++	++	++	-	-	-	++	++	++	-
12	C12	+++	+++	++	-	-	-	++	++	++	-
13	C13	+++	++	-	-	-	-	++	++	++	+
14	C14	+++	++	++	-	-	-	++	++	++	+
15	C15	+++	++	-	-	-	-	++	++	++	-
16	C16	+++	++	-	-	-	-	++	++	++	+
17	C17	+++	-	-	-	-	-	++	++	++	++
18	C18	+++	++	++	-	-	-	++	++	++	++
19	C19	+++	++	++	-	-	-	++	++	++	+
20	C20	+++	++	+	-	-	+	++	++	++	-
21	C21	+++	-	-	-	-	-	++	++	++	-
22	C22	+++	-	-	-	-	-	++	++	++	++
23	C23	+++	++	++	-	-	-	++	++	++	-
24	C24	++	-	-	-	-	-	++	++	++	-
25	C25	+++	++	+	-	-	-	++	++	++	+
26	C26	++	-	-	-	-	-	++	++	++	+
27	C27	++	+	-	-	-	-	++	++	++	+
28	C28	+++	+++	-	-	-	-	++	++	++	++
29	C29	+	+	-	-	-	-	++	++	++	-
12	C30	+++	++	++	-	-	-	++	++	++	++
31	C31	+++	++	+	-	-	-	++	++	++	+
32	C32	+++	++	++	-	-	+	++	++	++	-
33	C33	+++	++	+	-	-	-	++	++	++	-
34	C34	+++	++	++	-	-	-	++	++	++	-
35	C35	+++	++	+	-	-	-	++	++	++	-
36	C36	++	-	-	-	-	-	++	++	++	-

[a] *NaCl was used at indicated dose to adjust the salt concentration;*

[b] *NaOH(1N) and HCl(4N) were used to adjust the various pH of the growth medium;*

[c] *"+" or "-" sign indicate the growth of bacteria in a particular condition and number of + sign denoted the intensity of growth.*

Table 3. Biochemical properties of the bacterial isolates

Sl. no.	Isolates	Biochemical tests performed							
		Amylase	Catalase	Gelatine hydrolysis	Methyl red	H_2S production	Citrate utilization	Urease	Carbohydrate utilisation
1	C1	*+ve	+++ve	+ve	-ve	-ve	-ve	-ve	A, F
2	C2	+ve	+++ve	-ve	-ve	-ve	-ve	-ve	D, F ,MO, R
3	C3	+ve	+++ve	-ve	-ve	-ve	-ve	-ve	C,D,F,G,I,L,MO,R,S
4	C4	+ve	++ ve	-ve	-ve	-ve	-ve	+ve	A,C,D,F,G,MA,MO,R,S
5	C5	+ve	+++ve	+ve	+ve	-ve	-ve	-ve	–
6	C6	+ve	+++ve	-ve	-ve	-ve	-ve	+ve	A,C,D,F,G,L,MA,MO,R,S
7	C7	+ve	+++ve	-ve	-ve	-ve	-ve	-ve	F
8	C8	-ve	+ve	-ve	-ve	-ve	-ve	-ve	–
9	C9	+ve	+++ve	-ve	-ve	-ve	-ve	-ve	A,C,D,F,R,S
10	C10	+ve	+++ve	-ve	+ve	-ve	-ve	-ve	D,F
11	C11	+ve	++ ve	-ve	-ve	-ve	-ve	-ve	A,C,D,F,MO,R,S
12	C12	-ve	+++ve	-ve	+ve	-ve	+ve	-ve	–
13	C13	+ve	+ve	-ve	-ve	-ve	-ve	-ve	–
14	C14	-ve	++ ve	-ve	-ve	-ve	-ve	-ve	–
15	C15	+ve	+++ve	-ve	-ve	-ve	-ve	-ve	–
16	C16	-ve	+ve	-ve	-ve	-ve	+ve	-ve	–
17	C17	-ve	++ ve	-ve	-ve	++ve	+ve	+ve	A
18	C18	-ve	+++ve	+ve	-ve	-ve	+ve	-ve	D,F,G,MO
19	C19	-ve	-ve	-ve	-ve	-ve	+ve	-ve	D,F
20	C20	+ve	+ve	+ve	-ve	-ve	+ve	-ve	A,C,D,F,G,I,MO,S
21	C21	-ve	-ve	-ve	-ve	-ve	-ve	+ve	–
22	C22	-ve	-ve	+ve	-ve	-ve	-ve	-ve	A,C,D,F,G,I,L,MA,MO,R,S
23	C23	-ve	+++ve	-ve	-ve	-ve	-ve	-ve	A,C,D,F,G,I,L,MA,MO,R,S
24	C24	-ve	-ve	-ve	-ve	-ve	-ve	-ve	–
25	C25	+ve	+++ve	-ve	-ve	-ve	-ve	+ve	A,C,D,F,G,I,L,MA,MO,S
26	C26	+ve	-ve	-ve	-ve	-ve	-ve	-ve	A,D,F,MO
27	C27	-ve	+++ve	-ve	+ve	-ve	+ve	-ve	A,C,D,F,G,MA,MO,R,S
28	C28	+ve	+ve	-ve	+ve	-ve	+ve	-ve	–
29	C29	+ve	+++ve	-ve	-ve	-ve	-ve	-ve	A,D,F,MO
30	C30	+ve	+ve	-ve	-ve	-ve	-ve	-ve	A,C,D,F,G,L,,MO,
31	C31	-ve	+++ve	-ve	-ve	-ve	+ve	-ve	A,C,D,F,G,L,MA,MO,S
32	C32	-ve	+++ve	-ve	-ve	-ve	-ve	-ve	A,C,D,F,G,L,MA,MO,S
33	C33	+ve	+++ve	-ve	-ve	-ve	-ve	-ve	D,F,MO,S
34	C34	+ve	+++ve	-ve	-ve	-ve	-ve	-ve	A,C,D,F,I,MA,MO,R,S
35	C35	+ve	+ve	-ve	-ve	-ve	-ve	+ve	A,C,D,F,I,L,MA,MO,R,S
36	C36	-ve	++ ve	-ve	-ve	-ve	+ve	-ve	A,C,D,F,G,I,L,MA,MO,R,S

**"+" or "-" sign show the positive or negative reaction*

A=Arabinose,C=Cellobiose,D=Dextrose,F=fructose,G=Galactose,I=Inositol,L=Lactose,MA=Mannitol,MO=Moltose ,R=Rffinose,S=Sucrose