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RESEARCH ARTICLE

Isolation and Screening of Ethylene Producing Bacteria

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ABSTRACT

Soil and fruit samples were collected from agricultural fields and local fruit markets near Coimbatore north region. Yeast-potato extract medium was used for isolation of ethylene producing bacterium, 11 bacteria were isolated and purified for further analysis. Ethylene producing ability was considered for screening the isolates using the Gas chromatography system with the porapak-Q column and flame ionization detector. Out of 11, 7 isolates produced ethylene when L-methionine was supplemented as the precursor. These bacterial isolates were characterized morphologically and biochemically and tentatively identified as Bacillus, E-coli and Pseudomonas sp. The microorganisms producing ethylene provide a potential option to replace artificial ripening agents in the future.

Keywords: *Methionine, Ethylene producing bacterium, Gas Chromatography*.

INTRODUCTION

Ethylene is a ripening hormone, which brings about changes in the physiological nature of fruits like changes in the colour, aroma, sugar, and pectins, flavour, texture all takes place during ripening. In microorganisms, the ethylene production is induced in two different ways one of which is the ethylene forming enzymes (EFE) L-methionine is catalyzed to α-ketoglutarate then ethylene is produced by the addition of oxygen at the end of the cycle Freebairn and Buddenhagen (1964) stated that *Pseudomonas solanacearum* is capable of producing ethylene. Two hundred and twenty-nine ethylene-producing strains of bacteria were identified. Some of the examples for ethylene producing organisms are *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Lactobacillus plantarum*, *Corynebacteriumglutamicum*, *Enterobacterasburiae Enterobacteraerogenes Staphylococcus aureus*, *Micrococcus luteus, Bacillus subtilis, Serratiamarcescens, Aeromonas salmonicida* (Kazuhiro *et al*., 1992).

Ethylene is the ripening inducing hormone in climacteric fruits like papaya, mango, banana, sapota, custard apple, etc., Non-climacteric fruits are those which do not depend on ethylene for ripening (Chervin *et al*., 2008) Artificial ripening is a process of ripening fruits by hastening the process exogenously. The surplus usage of chemicals like calcium carbide renders the fruits unfit for consumption by altering its nature and quality, thus making it unfit for consumption due to the presence of harmful chemical traces like calcium chloride. This leads to problems like diarrhea, eye, throat irritation and other health complications. It can also turn carcinogenic and lead to neurological disorder in humans. Ethephon, an organophosphate, is used on fruits for inducing ripening. It is commercially available as ethrel, the liquid form of ethylene which is used as a pre-harvest ripening agent (Dhembare, 2013).

So far industrially manufactured pure ethylene gas is utilized in different ripening studies but the eco-friendly ethylene gas from microorganisms is not tested for its efficiency to trigger the ripening process of fruits (Liu *et al*., 2015). Bio-ethylene produced by microorganisms is eco-friendly and efficient when used in the ripening process compared to artificial ripeners(Khalid *et al*., 2006). Development of efficient bacterial consortium for ripening of fruits opens a new door in the field of ripening of fruits. Hence this study aims in isolating ethylene producing bacteria from different fruits and agricultural wastes for the consortium development.

MATERIAL AND METHODS

Isolation of ethylene producing microorganisms

Sample collection

Soil samples were collected from Agricultural College and Research Institute, Coimbatore - 641003 (11.01 52° N, 76.9326° E). The fruit samples were collected from the local fruit market and stored in the icebox for further usage.

Isolation

The medium used for isolation is YP(potato

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extract-yeast extract-mannitol) medium (Primrose, 1976) The composition of the medium includes potato (peeled)-200g, dextrose -20g, yeast extract -2g, agar-15g, distilled water-1000ml, pH-5.6. The soil and fruits samples were serially diluted and plated in YP plates and incubated at 28°C for 3d. The colonies were selected based on morphology, subcultured and a total of 11 pure cultures of the bacterial isolates were subcultured on Brown and Dilworth medium slants and stored at 4°C for further studies (Billington *et al*., 1979).

Characterization and screening of the Bacterial isolates

Morphological characterization

The morphological characters of the isolates were studied by following the method of Gerhardt (1981). The results are represented in Table 1.

Biochemical characterization

The biochemical characterization of the bacterial isolates was performed by the method of Holding (1971). The biochemical tests performed were indole test (Ahmed *et al*.,2005), methyl red test, citrate utilization test (Vaughn *et al*., 1939), starch hydrolysis, gelatin hydrolysis, catalase, urease (Clarke 1952). The results are displayed in Table 2.

Screening of Bacterial isolates for ethylene production

The screening of ethylene producers was done by means of the colorimetric assay (Larue and Kurz, 1973) and quantitative analysis (Kvpczynska *et al*., 2003). The quantitative analysis was executed through the gas chromatograph and the colorimetric analysis was performed by using a spectrophotometer.

Colorimetric assay for ethylene production

The 1 % (v/v) containing 10^8 CFU ml^{-1} of the starter culture was inoculated into the serum bottles of 100ml containing 30ml of Brown and Dilworth broth supplemented with 500mg L-methionine (Bird *et al*., 1974). The serum bottles were sealed with aluminum caps and kept for incubation at 30°C for 3 d and the ethylene production was assayed by taking the oxidant solution at a volume of about 1.5ml in 50ml serum bottles. The gas sample from the headspace of the ethylene-producing bacterial isolates was taken using the syringe and injected into the bottles containing oxidant solution and kept in a rotary shaker for vigorous shaking at 300 rpm for 90 min at 30°C. The caps were removed and 0.25ml of 4 N sulphuric acid and 0.25ml of 4 N sodium arsenite were added and kept for 1 h incubation. Development of dark yellow colour is the indication of positive ethylene production by the bacterial isolates (Table 4).

Quantitative assay for ethylene production

Gas chromatography is used for the detection of ethylene which can sense traces of molecules even at ppbv (parts per billion volume) range (Zaidi *et al*., 2016). The instrument used in this study was "Thermo GC-700" with porapak Q-column and FID (Flame ionization detector). The carrier gas used was nitrogen and the mobile phase was maintained at a flow rate of 30ml min^{-1}. The Brown and Dilworth broth was used for growing ethylene producing Microorganisms. The composition of the broth is Glucose-2.50g, Potassium dihydrogen phosphate-0.36g, Dipotassium phosphate-1.40g, Magnesium sulphate-0.25g, Calcium chloride-0.02g, Sodium chloride-0.20g, Ferric chloride-6.60g, EDTA-15mg, Zinc sulphate-0.16mg, Sodium molybdate-0.20 mg, Boric acid-0.25mg, Manganese(II)sulphate-0.20 mg, Copper sulphate-0.02 g, Cobalt chloride-1.00 µg, Methionine-500mg, Thiamine HCl-1.00 mg, Calcium pantothenate-2.0 mg, Biotin-1.00 µg, pH -6.5, Distilled water -1000 ml. All the trace chemicals were weighed in terms of milligrams and the stock solution was prepared for 100 ml and added to the medium in terms of required quantity of medium prepared. The serum bottles of 100 ml volume containing Brown and Dilworth broth of 30ml was inoculated with the isolates and L-methionine was added as the precursor and incubated for 4 days. By the end of the incubation period, the gas collected from the headspace was taken for GC analysis. The injector and column temperature is fixed at 80°C and 200°C for ethylene detection and the corresponding peak can be observed in the computerized system attached to the GC. The peak area of the gas injected and the Retention time (RT) could be visually seen on the screen.

Molecular characterization

The molecular characterization of ethylene producing bacteria was done after screening for ethylene production by means of both quantitatively and qualitatively. The best culture was taken for sequencing using 16S rDNA.

RESULTS AND DISCUSSION

Totally 11 isolates were isolated from the collected samples. Out of that 5 isolates were from fruit samples and the remaining 6 were from the soil. The isolates were purified and the morphological and biochemical characterization was done. Morphological characteristics like colony colour, topography, margin and Gram's reaction were observed for each bacterial isolate after 24 h of growth. The isolate EI 1 was Gram-negative with raised and erose margin with creamy white colour. EI 2 was found to be yellow coloured with an entire margin and raised colonies showing negative Gram's reaction. EI 3 was yellowish white with the flat colony,

Table 1. Morphological characteristics of the bacterial isolates

S.no	Isolates	Colony colour	Topography	Margin	Gram's reaction
1	EI1	Creamy white	Raised	Erose	-ve
2	EI2	Yellow	Raised	Entire	-ve
3	EI3	Yellowish white	Flat	Undulate	-ve
4	EI4	Creamy white	Raised	Undulated	+ve
5	EI5	Creamy white	Flat	Undulate	+ve
6	EI6	Creamy white	Flat	Entire	-ve
7	EI7	Yellow	Flat	Entire	-ve
8	EI8	White	Flat	Erose	-ve
9	EI9	Dull white	Flat	Erose	-ve
10	EI10	Dull white	Flat	Erose	+ve
11	EI11	White	Flat	Erose	-ve

+ positive –Negative

undulate margin and gram-negative. EI4 was a Gram-positive bacteria with creamy white, raised, and undulated margin. EI 5 and EI 6 were creamy white and Gram-negative. EI 7 was a yellow, flat entire colony and takes negative Gram's reaction. EI 8 is Gram-negative white coloured, flat and erosed margin colony. EI 9 and EI 10 were dull white, flat and erose with Gram-negative and Gram-positive reactions respectively. EI 11 was white Gram-negative with flat and erosed margin. The results were presented in Table 1 and Table 2, respectively.

Table 2. Biochemical characterization of the bacterial isolates

Isolates	Indole Production	MR	VP	Citrate Utilization	Starch Hydrolysis	Gelatin Hydrolysis	Catalase	Urease
EI 1	_	_	+	+	+	+	+	+
EI 2	_	_	+	+	_	+	_	_
EI 3	_	_	+	+	+	+	+	+
EI 4	_	_	+	+	+	_	+	_
EI 5	+	_	+	+	+	+	+	+
EI 6	+	_	+	+	+	_	_	_
EI 7	+	+	_	+	+	+	+	_
EI 8	+	+	_	+	_	_	+	_
EI 9	_	+	_	+	+	_	+	+
EI 10	_	_	+	_	+	_	_	_
EI 11	+	_	+	+	+	+	+	_

+ positive – Negative

The biochemical characterization like indole production, methyl-red, Voges-Proskauer, citrate utilization, starch hydrolysis, gelatin hydrolysis, catalase and urease were done for the isolates.

The isolates EI 1, EI 2, EI 3, EI 4, EI 9 and EI 10 showed negative indole production. The isolates EI 5, EI 6, EI 7, EI 8 & EI 11showed positive result which indicates the production of tryptophanase

Table 3. Quantification of ethylene produced by the isolates.

Isolates	nmol.L^{-1}
EI1	460
EI 2	-
EI 3	440
EI 4	360
EI 5	-
EI 6	570
EI 7	-
EI 8	388
EI 9	363
EI 10	385
EI 11	110

Table 4. Qualitative assay for ethylene production

Isolates	Colour change
EI1	+
EI 2	-
EI 3	-
EI 4	+
EI 5	-
EI 6	+
EI 7	-
EI 8	+
EI 9	-
EI 10	+
EI 11	+

+ Development of Straw yellow colour - No colour development

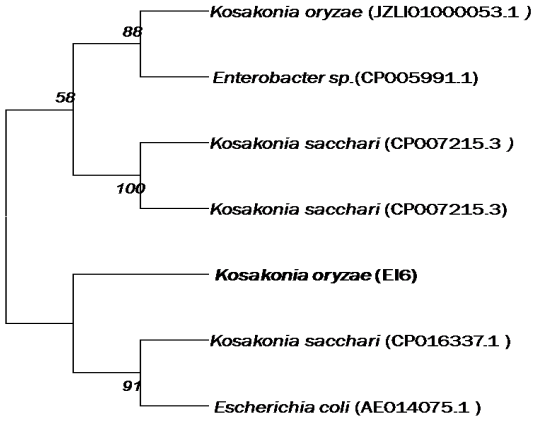

Figure 1. Phylogenetic relationships in Kosakonia; neighbour joining phylogenetic tree constructed with bootstrap value 100 using Mega.7

having the ability to digest tryptone resulting in indole production by the bacterial isolates. Almost all the isolates have the starch hydrolysis ability due to the presence of amylase production capacity.

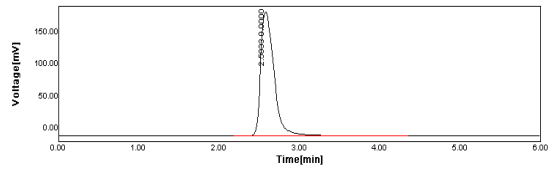

Figure 2a.The GC chromatogram of standard ethylene

Most of the ethylene-producing bacterial isolates were Gram-negative and also they showed a positive result for catalase and urease which indicates the positive correlation for ethylene production (Yang *et al*., 1967). From the obtained results the isolates may tentatively belong to *Pseudomonas*, *E.coli* and *Bacillus* genera. These results were in accordance with the experiments done by Primrose and Dilworth 1976 as they have also used L-methionine as the precursor for the ethylene production in *E-coli* (Primrose and Dilworth 1976).

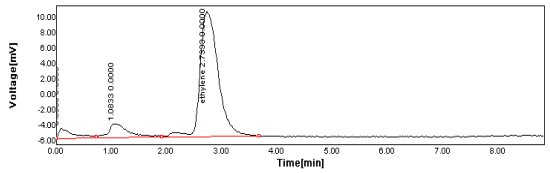

Figure 2b. Chromatogram of ethylene producing bacterial isolate.

The colorimetric assay for ethylene production indicates the production of straw yellow colour as a positive reaction. The isolates namely EI1, EI4, EI6, EI8, EI10, EI11 showed positive results (Laure *et al*., 1973) (Table 4). The quantitative assay by gas chromatography is the most accurate method for ethylene detection and quantification (Zaidi *et al*., 2016). The samples were run for confirmation of ethylene production in GC. The retention time (RT) of the standard ethylene gas was obtained at 2.5 min. Then the samples which have to be tested for ethylene production were run and the production of ethylene gas by the isolates was obtained in the near retention time. This shows the production of ethylene gas by isolates and the corresponding peak percentage was obtained. Based on the peak values and the retention time the isolates were screened for the production of ethylene. The isolates that are exhibiting positive ethylene production were listed in Table 3. Totally 7 isolates showed ethylene production at desirable levels namely EI1 460 nmol.L^{-1}, EI3 440 nmol.L^{-1}, EI4 360 nmol.L^{-1}, EI6 570 nmol.L^{-1}, EI8 388nmol.L^{-1}, EI9 363nmol.L^{-1}, EI10-385 nmol.L^{-1} and EI11-110 nmol.L^{-1}.The peak obtained for standard ethylene which is 99.5 % pure ethylene gas and from the samples are represented in Figure 1a and Figure 1b, respectively (Table 3). The isolate EI6 exhibiting maximum ethylene production is taken for sequencing and having 1452 basepairs it showed 99.06% of identity and having a close relationship with *kosakonia oryzae* (Figure1).

CONCLUSION

In the present study, a total of 7 isolates were screened and tested for their ethylene production. Integrative approaches combining biochemical and genetic experiments can enhance ethylene production. Thus, the bio-ethylene produced by microorganisms can be utilized as an alternative source to chemically produced ethylene.

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