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UTILIZATION OF WASTE AGAR GENERATED IN PLANT TISSUE CULTURE LABORATORY FOR PRODUCTION OF GALACTOSE BY AGAROLYTIC BACTERIA AND THEIR ENZYME AGARASE

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ABSTRACT

Large amount of waste agar is generated in plant tissue culture units. In the present investigation, agarolytic bacteria isolated from enrichments of dumped waste agar and soil collected from a plant tissue culture laboratory were used to hydrolyze waste agar to obtain value added product like galactose. Out of 35 microbial cultures, seven cultures could hydrolyze waste agar yielding galactose in the range of 39 to 63 mg/kg waste agar. Biochemical characterization revealed their identification as species of *Enterobacter*, *Micrococcus* and *Serratia*, being reported for the first time. The two selected strains of *Enterobacter sp.* were studied for the production of enzyme agarase and maximum enzyme activity was 0.97 U/ml/min using nutrient broth with 0.2% waste agar, shaking culture condition, cell density 10^9 cells/ml, incubation temperature 35°C and incubation period of 48 h. The studies pointed out the potential of bacterial isolates for conversion of waste of plant tissue culture industry into galactose, a product with commercial value as well as production of enzyme; agarase.

KEY WORDS: Agarolytic bacteria, Plant tissue culture laboratory, Waste agar, Agarase, Galactose.

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INTRODUCTION

Agar is a jelly like substance obtained from a polysaccharide which gets accumulated in the cell walls of red algae namely the genera *Gelidium*, *Gracilaria* and *Sphaerococcus*. It is a complex polysaccharide, composed of two fractions, agarose and agaropectin where agarose is the major constituent of agar. Agar is used throughout the world to provide a solid surface for preparation of growth of microorganisms[1-3] especially in microbiological and pathological laboratories and plant tissue culture laboratories (PTCLs) for growing seedlings since it forms clear, stable and firm gel. Numerous plant tissue culture laboratory laboratories supply a variety of plants in large volumes and their number is increasing throughout the world. In India >100 PTCLs are engaged in plant propagation programme and ~25% are in Maharashtra State. Once the plants are ready for sowing in soil, the seedlings from agar medium are detached and the residual agar with low nutrient contents thus becomes a waste agar. Around 1500 kg waste agar is generated per day in a medium sized PTCL which poses a problem of disposal. Agarolytic bacteria are capable of utilizing agar as a sole source of carbon. In the literature, agarolytic activity has been reported in various microorganisms[4-16]. These bacteria produce agarase, the enzyme system hydrolyzing agarose into galactose as an end product. Production of agarase by these microorganisms is reported by some researchers[17-23]. The present paper describes exploration of the agarolytic microbial cultures for hydrolyzing waste agar to obtain galactose, one of the industrially important carbohydrates as a by-product and the enzyme agarase.

MATERIALS AND METHODS

1. Collection of waste agar and soil samples

Waste agar (WA) was collected from a plant tissue culture laboratory situated at Manjari, Pune, MS, India and stored in a cold room. The agar content of waste agar was 0.8%. Waste agar dumped in the pits and the soil samples in the vicinity of pits were collected for isolation of agarolytic microorganisms following enrichment technique.

2. Isolation, selection and identification of the agarolytic bacteria

Presterilised 100 ml Glucose Yeast extract Peptone (GYP) medium containing 1 g of waste agar (since waste agar contains 0.8 g% agar, final concentration of agar in the medium was 0.008 g% which served as substrate) was inoculated with 10 g of waste agar (as inoculum) along with soil and incubated at ambient temperature (28±2 °C) on orbital shaker (Steelmate, India) at 100 rpm for one week. 100 g waste agar was added to 100 ml each GYP broth (GYPWA) and Davis Mingioli's synthetic medium (DMSWA) separately so as to have agar concentration as 0.8 g% in each medium, sterilized at 121 °C for 20 min, and dispensed in 20 ml aliquots in presterilised glass containers. The media were then inoculated with 10 ml enrichment cultures, incubated at ambient temperature (28±2°C) up to 72 h and observed for the liquefaction of the agar medium as compared to uninoculated control. The enrichments showing promising liquefaction activity in DMSWA were used for isolation of agarolytic microorganisms using Nutrient agar (NA, Hi Media, India) plates by streak plate method and incubated at ambient temperature (28±2 °C) for 48 h. The well isolated and morphologically distinct colonies were maintained on NA slants at 4°C for further studies.

For checking the agarolytic efficiency of the individual isolates, one ml suspension of the 24 h grown individual bacterial culture having cell density as 10^7 cells/ml was inoculated in 20 ml of GYPWA medium and incubated upto one week at 30°C under stationary culture condition. The agarolytic activity of the microorganisms was evidenced by appearance of gas bubbles and liquefaction of the agar medium. Since the hydrolysis of agar due to bacterial activity results in the release of galactose, agarolytic activity estimated in terms of galactose released in the medium using DNSA method (Miller[24]) and expressed as mg/l. Based on the visual observations for extent of hydrolysis of GYPWA and the amount of galactose released during hydrolysis by all the isolates, seven isolates

were selected for further studies. Identification of the selected seven isolates was performed using conventional morphological, physiological and biochemical tests according to Cruickshank[1] and Krieg and Holt[25].

3. Hydrolysis of waste agar using enzyme preparation

The selected seven microbial cultures were inoculated in 100 ml nutrient broth in 250 ml Erlenmeyer flasks and incubated under shake culture condition at 100 rpm on orbital shaker at 30°C (Amerex, USA) for 24 h. The culture broth was centrifuged at 8000 rpm for 20 min at 4°C using refrigerated centrifuge (Kubota 6930, Japan) and cell free supernatant (CFS) was considered as crude enzyme for hydrolysis of waste agar. 80 ml CFS thus obtained was used for partial purification of enzyme using ammonium sulphate at 70 % saturation. The precipitate was collected by centrifugation at 8,500 rpm at 4 ºC in a refrigerated centrifuge. The clear supernatant was decanted, the precipitate was dissolved in 3 ml 20 mM Tris HCl buffer to serve as partially purified enzyme (PPE) and used for hydrolysis of waste agar. 20 ml sterilized GYPWA medium was inoculated with 5 ml of CFS and PPE suspended in 20 mM Tris HCl buffer separately, incubated at 30°C and checked for hydrolysis of waste agar. The agarase enzyme assay was performed using the protocol given by Vijayraghavan and Rajendran[23].

Enzyme assay: A mixture of 2 ml of 0.25 g% agarose (Sigma, USA) dissolved in 20 mM Tris HCl buffer (pH 8.0) and 1ml crude enzyme was incubated for 2 h at 30°C. After an appropriate incubation period of 2 h, the tubes were kept in boiling water bath for 15 min to stop the reaction. For estimation of galactose, each tube containing 1 ml of enzyme reaction mixture + 1 ml DNSA reagent was kept in boiling water bath for 10 min and allowed to cool. The volume was made upto 10 ml using distilled water. The absorbance was measured at 540 nm using boiled enzyme as blank**.** One unit of the agarase enzyme activity was defined as the amount of protein per ml producing µmol of reducing sugar as D-galactose per minute under condition of the assay.

4. Production of enzyme under different environmental conditions:

Based on the visual observations of the waste agar hydrolysis by CFS and partially purified enzyme, two isolates were selected for enzyme production under different environmental conditions. The optimization of enzyme production was performed in the similar way as described by Mehta *et al*.[26] for keratinase and Jholapara *et al*.[27] for production of chitinase. In the present paper the agarase production was carried out as detailed below by changing one parameter at a time and keeping other parameters constant as follows:

- Media used - NB, GYE, NB+ 0.2 g/l waste agar, GYE+ 0.2 g/l waste agar, Production medium S (PMS)[22] and Production medium VR (PMVR)[23]
- Culture conditions - shaking, stationary
- Cell density of the inoculum -10^6, 10^7cells/ml, 10^8cells/ml and 10^9cells/ml
- Temperature of incubation - 25 °C, 30 °C, 35 °C and 40 °C
- pH of the medium - 6, 7, 8 and 9
- Incubation period - 0 h, 24 h, 48 h, 72 h and 96 h

Initially for selection of a suitable medium for production of agarase enzyme, 100 ml of each medium mentioned above was inoculated with 4 ml cell suspension of each isolate having cell density as 10^8 upto 48 h keeping uninoculated control of respective medium. After 48 h, the culture broths were centrifuged at 8000 rpm for 20 min at 4ºC using refrigerated centrifuge and the cell free supernatant (CFS) was used for the enzyme assay as described earlier. The medium exhibiting maximum enzyme activity was selected for further studies on optimization of other parameters. The production of an enzyme under all optimum conditions was also carried out.

RESULTS AND DISCUSSION

1. Hydrolysis of waste agar

The performance of cultures when grown in GYPWA medium containing waste agar is seen in Fig. 1. It could be seen that there was a rise in volume due to formation of gas

bubbles leading to cracking the agar bed. These observations are indicative of liquefaction of agar concomitant with the development of bacterial density and bubbles.

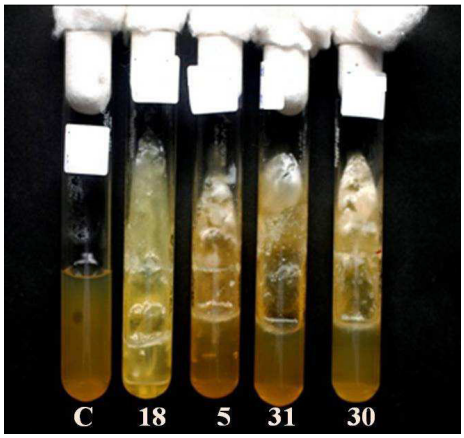

Figure 1.Hydrolysis of waste agar incorporated in GYPWA Medium by the bacterial isolates # 18, 5, 31, 30

2. Isolation and selection of agarolytic microorganisms

In all, 35 morphologically distinct isolates were obtained from the enriched samples inoculated on Nutrient agar. The visual observations on the extent of agar hydrolysis using GYPWA medium by selected isolates and amount of galactose released are presented in Table 1. Out of 35 microbial cultures, seven cultures could hydrolyze waste agar yielding galactose. Isolate Nos. 5, 8, 9, 18, 28, 30, and 31 showed noticeable agarolytic activity in the form of vigorous bubbling in the medium, complete liquefaction of agar or formation of cracks in the medium. The amount of galactose released in the medium by these isolates was > 30 mg/l i.e. in the range from 39 – 63 mg/l. From the set of cultures screened for agarolytic activity, seven cultures were selected for further studies on the basis of galactose released. Thus, maximum 63 mg of galactose could be obtained from 1 kg of waste agar.

Table 1
Agar hydrolysis by the selected bacterial isolates

Isolate No	Visual observation on waste agar hydrolysis in GYPWA	Galactose, mg/l
5	Complete liquefaction of the medium	59.00
8	Vigorous bubbling of the medium	49.67
9	Complete liquefaction of the medium	39.22
18	Cracks in the medium and vigorous bubbling of the medium	63.42
28	Growth at the surface and liquefaction of the medium	42.11
30	Complete liquefaction and slight cracking of the medium	44.43
31	Complete liquefaction of the medium	57.86
Control	No change in the consistency of the medium and no growth	2.73

3. Hydrolysis of waste agar using enzyme preparation

The results on galactose released by CFS and PPE are depicted in Fig .2. It was observed that out of seven isolates under study, two isolates # 18 and 30 have shown maximum agarolytic activity in terms of release of galactose using CFS and PPE.

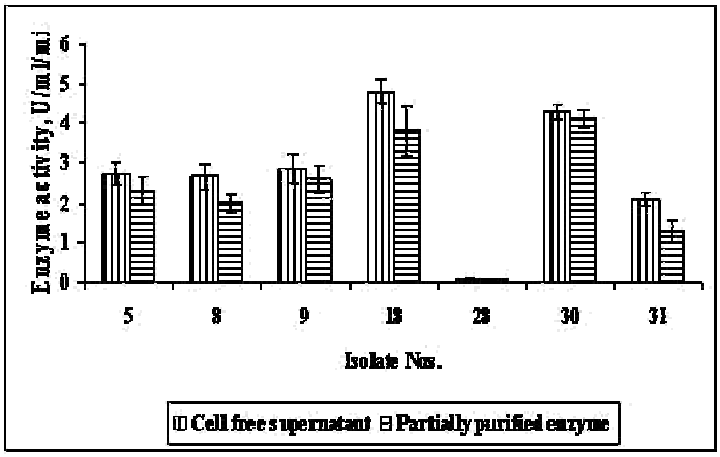

Figure 2.*Enzyme activity of cell free supernatant (CFS) and partially purified enzyme (PPE) of the selected isolates*

4. Identification of the microbial cultures under study

Based on morphological, physiological and biochemical tests, the selected 7 microbial cultures viz. the Isolate Nos. 5, 8, 9, 18, 28, 30, and 31 showing noticeable agarolytic activity were biochemically identified as detailed in Fig. 2. The results showed that out of seven microbial cultures under study, three isolates were identified as *Enterobacter cloacae,* two isolates as *Enterobacter aerogenes,* one isolate each as *Micrococcus* sp. and *Serratia marcescens.*

Table 2
Identification of the selected isolates based on biochemical characterization

Isolate No.	Identification
5	*Enterobacter cloacae*
8	*Enterobacter cloacae*
9	*Enterobacter aerogenes*
18	*Enterobacter aerogenes*
28	*Micrococcus* sp.
30	*Enterobacter cloacae*
31	*Serratia marcescens*

In the literature, some agarolytic microorganisms and their ability to produce agarase enzyme have been reported e.g. *Vibrio*[9],*Paenibacillus*[10], *seudoalteromonas*[11], *Zobellia*[20],*Simiduia*[12],*Agarivorans*[21], *Aliagarivorans*[13], *Cellvibrio*[14-15] *Bacillus*[22] and myxomycete *Fuligoseptica*[7]. Most of the agarolytic microorganisms were isolated from the marine environment. In the present investigation, the agarolytic bacteria are isolated from soil and dumped waste agar from neutral soil samples. Meager data are available the isolation of agarolytic bacteria belonging to the genera *Enterobacter*, *Micrococcus* and *Serratia.* This seems to be the first report on the agarolytic activity of the isolates belonging to these three genera isolated from soil.

5. Production of enzyme under different environmental conditions

Based on the results of agar hydrolysis using CFS, two isolates belonging to the genus Enterobacter i.e. *Enterobacter aerogenes* (Isolate #18) and *Enterobacter cloacae* (Isolate #30) showing maximum agarolytic activity both by cell free supernatant (CFS) and partially purified enzyme (PPE), were selected for the production of enzyme under different environmental conditions . The results on optimum conditions for production of enzyme, thus obtained and the enzyme production under all optimum conditions are detailed in Table 3. The results showed that

the enzyme activity was maximum when initial pH of the medium was 7 by both the isolates under study. The enzyme activity by the Isolate 18 and that of Isolate 30 was 0.97 U/ml/min under all optimum conditions. There was slight increase in the activity of Isolate 18 from 0.94 to 0.97 U/ml/min and that of Isolate 30 was increased from 0.84 to 0.97 under all optimum conditions as compared to the enzyme activity at pH 7.

Table 3
Enzyme production under different environmental conditions

Optimum condition	Enzyme activity, units/ml/min	
	Isolate #18	Isolate #30
Medium, NB + 0.2% waste agar	0.55	0.60
Culture condition, Shaking	0.17	0.13
Cell density, 10^9cells/ml	0.77	0.76
Temperature, 35 °C	0.68	0.75
pH, 7	0.94	0.84
Incubation period, 48 h	0.58	0.47
Under all optimum conditions	**0.97**	**0.97**

CONCLUSION

Agarolytic bacteria were isolated from soil by an enrichment technique using waste agar as the substrate. Promising liquefaction, cracking and the bubbling was seen in both GYPWA and DMSWA medium by 7 isolates. Maximum 63 mg of galactose could be obtained from 1 kg of waste agar. Maximum enzyme production was observed by the isolate *Enterobacter aerogenes* and *Enterobacter cloacae* as 0.97 U/ml/min under the optimum conditions i.e. in the NB + 0.2 % waste agar medium of pH 7, under shaking condition, at cell density of inoculum as 10^9 cells/ml, incubation temperature of 35 °C for 48 h.

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