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Full Length Research Paper

Use of psyllium (isubgol) husk as an alternative gelling agent for the culture of prokaryotic microalgae (Cyanobacteria) *Chroococcus limneticus* Lemmermann and eukaryotic green microalgae (Chlorophyta) *Scenedesmus quadricauda* (Turpin) Brebisson

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Agar is popularly used as gelling agent to obtain *in vitro* culture of prokaryotic and eukaryotic microalgae. Exclusive use of agar is resulting in over exploitation of its resources and makes it essential to look for alternative and cheap sources. The study reports *in vitro* culture of prokaryotic microalgae (Cyanobacteria) *Chroococcus limneticus* and eukaryotic green microalgae (Chlorophyta) *Scenedesmus quadricauda* on BG11 medium solidified with 15 g L^{-1} agar or 10, 15, 20, 25, 30, 35 and 40 g L^{-1} psyllium (isubgol) husk as gelling agent. The results demonstrated that prokaryotic microalgae *C. limneticus* was less appropriate for culture on psyllium husk gel compared to eukaryotic green microalgae *S. quadricauda*. However, the cells of *C. limneticus* could be multiplied on 10 or 15 g L^{-1} psyllium husk as gelling agent. The highest number of 642000 cells mL^{-1} of *C. limneticus* on 15 g L^{-1} psyllium husk were far lower than 722000 cells mL^{-1} on agar gelled medium (control). Contrarily, the cells of *S. quadricauda* could be multiplied on all concentrations of psyllium husk. Compared to the highest number of 546000 cells mL^{-1} of *S. quadricauda* on 15 g L^{-1} agar (control), the highest number of 538000 cells mL^{-1} was recorded on 15 g L^{-1} psyllium husk gel. The results indicated very similar and comparable cell multiplication behavior of *S. quadricauda* on agar or 15 g L^{-1} psyllium husk and demonstrate the advantage of the very cheap alternative gelling agent psyllium husk over expensive agar for possible use in analytic experiments pertaining to eukaryotic green microalgae.

Key words: Agar-gelled medium, *in vitro*, psyllium husk gelled medium, viscosity.

INTRODUCTION

Several gelling agents have been described for obtaining *in vitro* cultures of blue-green algae. Among different gelling agents agar is most extensively used to culture the microalgae (Atici et al., 2006). Agar is being used to culture microbes and plants since long after its introduction more than 100 years ago (Pelczar et al.,1986), as it is highly stable, clear and resistant to metabolic changes during culture (McLachlan, 1985; Henderson and Kinnersley, 1988). Exclusive use of agar is resulting in over exploitation of its source plants- kelp marine plants. The demand for use of agar in scientific work is continuously on increase in the world. This increased demand and declining supply (with the passage of time) has resulted in increase in the prices of the agar, due to which scientific work in the poor and under developed countries is facing considerable constraints. A wide range

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Abbreviation: kPa: Kilopascal.

Table 1. Multiplication of prokaryotic microalgae *C. limneticus* cells on different concentrations of psyllium (isubgol) gelled media.

Day	Number of cells mL^{-1} of *C. limneticus*							
	Agar gel	Psyllium gel						
	15 g L^{-1} (Control)	10 g L^{-1}	15 g L^{-1}	20 g L^{-1}	25 g L^{-1}	30 g L^{-1}	35 g L^{-1}	40 g L^{-1}
1	424000	386000	204000	No multiplication of cells				
2	568000	462000	320000					
3	645000	502000	426000					
4	722000	614000	642000					

of gelling agents including carrageenans (Liners et al., 2005), alginates (Onsoyen, 1996), ficoll (Kao, 1981) agarose (Johansson, 1988) elrite (Lin and Casida Jr, 1984), starches (Tiwari and Rahimbaev, 1992; Nene et al., 1996), gum katira (Jain and Babbar, 2002) and xanthan gum (Babbar and Jain, 2006) have been tried as substitutes of agar with variable degree of success. Similarly, usefulness of psyllium or isubgol, a cheap gelling agent, for plant tissue culture medium (Babbar and Jain, 1998; Jain and Babbar, 2005) and microbial culture medium (Jain et al., 1997; Sahay, 1999) has also been established. The wide distribution and cultivation of its source plant, *Plantago ovata* for medicinal purposes in many countries, can prove to be an added advantage.

None of the experiments described effects of psyllium to maintain *in vitro* microalgal cultures. Therefore, the study was planned to determine if psyllium could act as alternative gelling agent to culture prokaryotic microalgae (Cyanobacteria) *Chroococcus limneticus* Lemmermann and eukaryotic green microalgae (Chlorophyta) *Scenedesmus quadricauda* (Turpin) Brebisson for analytical experiments.

MATERIALS AND METHODS

Microorganism and Medium

C. limneticus and *S. quadricauda* used in the experiment were collected from the Gazi Microalgae Collection Centre, (GAZI-MACC), Department of Biology Education, Gazi University, Ankara, Turkey. The cells of each microalgae were initially grown in separate shake flasks containing sterilized 100 mL each of BG 11 medium [(NaNO3, 15; K2HPO4, 0,4; MgSO4.7H2O, 0,75; CaCl2.2H2O, 0,36; citric acid, 0,06; iron (III) ammonium citrate, 0,06; Na2-EDTA, 0,01; Na2CO3, 0,2 g L^{-1}, 1 mL; trace elements solution, (H3BO3, 61; MnSO4.H2O, 169; ZnSO4.7H2O, 287; CuSO4.5H2O, 2,5; (NH4)6Mo7O24.4H2O, 12,5 mg L-1) pH: 6,8] commonly used for growing prokaryotic and eukaryotic green microalgae (Rippka, 1989) under light intensity of 30 000 lux (Philips 40 w fluorescent tube, Turkey) at 24 ±1°C for 25 days, in 12-12 h light-dark photoperiod. This medium contains only trace amounts of metal ions and allows rich growth.

Quantification and determination of microalgae cells

C. limneticus and *S. quadricauda* suspensions (200 µl) were aseptically cultured *in vitro* on 35 mL of BG11 medium solidified with 15 g L-1 bacteriological grade agar – control (Sigma Aldrich, St Louis, Missouri-here in after called agar) or 10, 15, 20, 25, 30, 35 and 40 g L^{-1} psyllium husk (isubgol- here in after called psyllium) (Marhaba Laboratories®, 142 Main Industrial Estate, Kot Lakhpat, Lahore, Pakistan) contained in Petri dishes (100 x 10 mm) using a sterile bent glass rod. The agar or psyllium was added after adjusting pH of the media to 6.8 with 0.1 N KOH or 0.1 N HCl before autoclaving, at 121°C, under pressure of 118 kPa for 20 min. Each concentration of psyllium including control was replicated 3 times. These plates were sealed with stretch film and incubated in an inverted position under light intensity of 30 000 lux (Philips 40 w fluorescent tube, Turkey) at 24 ±1°C, in 12 - 12 h light-dark photoperiod (Sanyo Versatile Environmental Test Chamber). The cell density of the respective microalgae was determined by counting colonies grown on the surface the spread-plate (Stainer et al., 1971) and was quantified using a Neubauer hemocytometer (Guillard, 1978; Cirik and Gokpinar, 1999). All cultures were examined daily for growth. The cell counting started seven days after initial culture during log phase and was done for four days without any break until the cells entered transitional phase.

RESULTS

Prokaryotic microalgae (Cyanobacteria) *C. limneticus*

A sharp difference was observed in the colony development behavior of *C. limneticus* on agar (15 g L^{-1}-control) or any concentration of psyllium as gelling medium (Table 1). Compared to agar gelled medium, lower number of colonies were recorded only on BG11 medium contained in 10 or 15 g L^{-1} psyllium as gelling agent on first, second, third or fourth log day (Figure 1). No colony development was recorded on BG 11 medium on 20-40 g L^{-1} psyllium as gelling agent. The results further showed that 386000 cells mL^{-1} on 10 g L^{-1} and 204000 cells mL^{-1} on 15 g L^{-1} psyllium as gelling agent on first day increased to 462000 and 320000 cells mL^{-1} on 2nd day; 502000 cells mL^{-1} and 426000 cells mL^{-1} on third day and 614000 and 642000 cells mL^{-1} on 4th day of culture. Comparing the cell multiplication behavior on 10 and 15 g L^{-1} psyllium gel medium, the number of cells mL^{-1} surpassed on agar gelled medium on all log days. The maximum number of 722000 cells mL^{-1} recorded on 4th log day on agar gelled medium were significantly higher compared to maximum number of 642000 cells mL^{-1} on 15 g L^{-1} psyllium gelled medium.

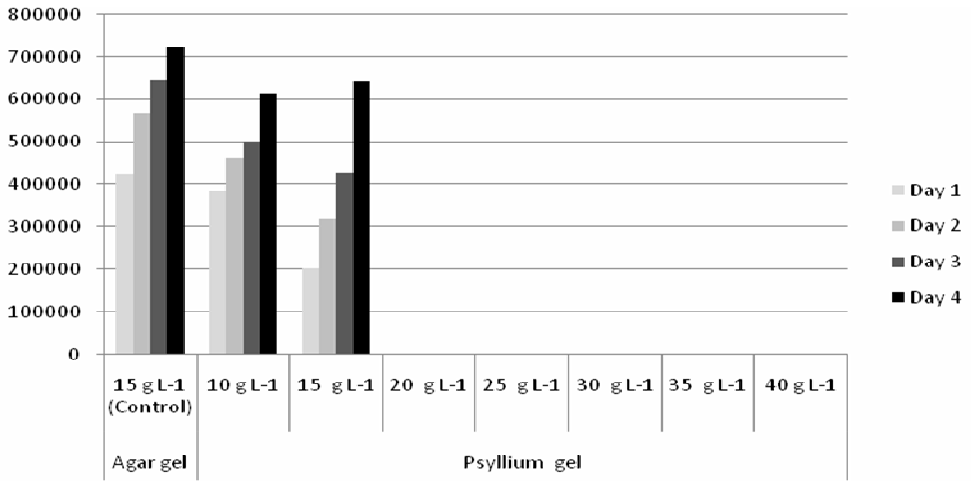

Figure 1. Schematic representation of the rates of multiplication of prokaryoticmicroalgae *C. limneticus* cells on different concentrations of psyllium (Isubgol) gelled media.

Table 2. Multiplication of eukaryotic green microalgae *S. quadricauda* cells on different concentrations of psyllium (Isubgol) gelled media.

Day	Number of cells mL^{-1} of *S. quadricauda*							
	Agar gel	Psyllium gel						
	15 g L^{-1} (Control)	10 g L^{-1}	15 g L^{-1}	20 g L^{-1}	25 g L^{-1}	30 g L^{-1}	35 g L^{-1}	40 g L^{-1}
1	88000	16000	120000	60000	32000	16000	16000	28000
2	100000	24000	128000	80000	120000	20000	36000	40000
3	400000	32000	386000	100000	150000	210000	54000	52000
4	546000	46000	538000	120000	154000	220000	62000	58000

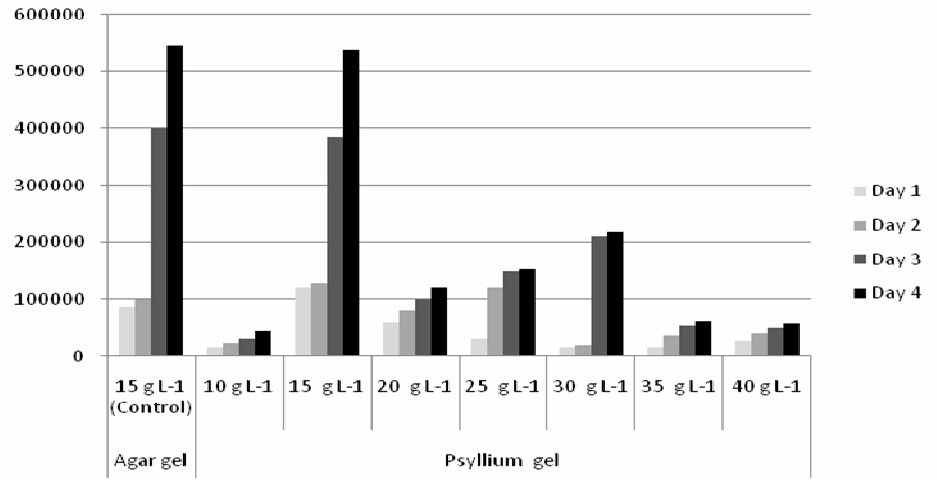

Figure 2. Schematic representation of the rates of multiplication of eukaryotic green microalgae *S. quadricauda* cells on different concentrations of psyllium (isubgol) gelled media.

Eukaryotic green microalgae (Chlorophyta) *S. quadricauda*

Different concentrations of psyllium as gelling agent had apparently different effects on the growth of *S. quadricauda* (Table 2, Figure 2). Very poor cell multiplication of 16000 cells mL^{-1} was recorded on BG11 medium containing 10 g L^{-1} psyllium as gelling agent on first log day. Compared to 88000 cells mL^{-1} on 15 g L^{-1} agar gelled BG 11 medium (control) - 15 g L^{-1} psyllium gelled BG 11

medium showed 120000 and 128000 cells mL^{-1} on first and 2nd log day. Thereafter, on third and 4th log day cell growth of 400000 and 546000 cells mL^{-1} was recorded on agar (control-15 g L^{-1}) and 386000 and 538000 cells mL^{-1} on 15 g L^{-1} psyllium gelled media. This shows comparable and similar cell multiplication behavior of both gelling agents. All other gelling concentrations of psyllium showed an increase in the *S. quadricauda* cells mL^{-1} in ascending order. However, the number of cells mL^{-1} recorded on all other concentrations of psyllium were lower compared with agar on first, second, third or fourth log days.

DISCUSSION

There is an extensive literature on culture techniques, maintenance conditions (e.g. Stein, 1973; McLellan et al., 1991; Becker, 1994) and gelling agents. Medium composition (e.g. diatoms require the inclusion of a silica source; many marine algae require vitamins) and strength of gelling agent (agar, agarose, bacto agar etc.) depends both on the requirements of the algae and the preferences of the researcher. Information on medium and gelling agent suitability along with full recipes are listed in the catalogues of all the major protistan culture collections e.g. CCAP Catalogue (http://www.ife.ac.uk/ccap, Tompkins et al., 1995).

The simplest method of maintenance of microalgae is by serial transfer from staled to fresh solid or liquid media and storage in the most suitable conditions for the individual isolate. Many microalgae can be maintained in this way for years by growth on suitable media. Successful maintenance is dependent upon transfer from well-developed parts of the culture, taking care to ensure that contaminants or genetic variants do not replace the original strain. Like agar, psyllium mucilage is colloidal and polysaccharidic in nature and is mainly composed of xylose, arabinose, and galactouronic acid. Rhamnose and galactose have also been reported. Two polysaccharide fractions have been separated from the mucilage. One fraction (eq wt. 700; uronic acid 20%) is soluble in cold water and upon hydrolysis yields D-xylose (46%), aldobiouronic acid (40%), L-arabinose (7%), and an insoluble residue (2%). The other fraction (eq wt 4000; uronic acid 3%) is soluble in hot water, forming a highly viscous solution which sets to gel when cooled and yields upon hydrolysis D-xylose (80%), L-arabinose (14%), alsobiouronic acid (0.3%) and traces of D-galactose (Laidlaw and Percival 1949, 1950).

It was observed that the psyllium was highly mucilaginous, sticky and contained husk particles, which remained suspended after autoclaving and solidification. The husk particles could be easily removed by purifying the psyllium to get mucilage devoid of husk. These husk particles seemed to have no inhibition on the gelling ability of psyllium. Bacteriological agar gels at 35°C and solidifies at 79.8°C (Sengor, 2001). This is desirable feature since it does not require quick dispencing after autoclaving making it suitable for adding heat labile supplements before pouring. Contrarily higher melting point (>100°C) and very narrow critical temperature (90-95°C) of solidification (Sahay, 1999) of psyllium necessitated quick dispensing of psyllium after autoclaving. However, no softening of the psyllium was observed thereafter, which indicated that there was no problem of metabolizing of psyllium during the course of experiment/s.

The genotypic changes in algae were not expected because algal cultures were grown with normal life regulatory factors such as nutrient medium, light source, and growth temperature, during the course of experiment. It was expected that basic structural differences between prokaryotic microalgae *C. limneticus* and eukaryotic green microalgae *S. quadricauda* and the growth requirements of both should make them respond in different ways during culture on psyllium or agar gelled media. It was also expected that the strength of gelling agent will affect the quantitative development of both cultures. Therefore, standardization of media formulae including gel strength was very important. As expected, the results showed that prokaryotic microalgae *C. limneticus* were less appropriate for culture on psyllium compared to eukaryotic green microalgae *S.quadricauda*.

Initial experiments in the laboratory (results not shown), showed that viscosity of psyllium gel at concentrations lower than 10 g L^{-1} was too low and 30 or 50 g L^{-1} was too high to favor culture development. Use of higher strength (3-5%) of psyllium (isubgol) as gelling agent resulted in difficult to pour, very viscous and sticky mass of gel. Previous researchers Sahay (1999) used 5% and Babbar and Jain (1998) used 3% psyllium for gelling media. Psyllium concentration as low as 1.5% (15 g L^{-1}) was found optimum to give a gel of good quality for *S. quadricauda* (and partially good gel for *C. limneticus*), without any adverse effects on cell multiplication quantitatively or qualitatively.

Low number of *C. limneticus* cell development on BG 11 medium at 10 or 15 g L^{-1} psyllium concentrations might be due to lower metabolic activity of the microalgae on psyllium gelled media. No cell multiplication of *C. limneticus* and lower number of cell multiplication of *S. quadricauda* on BG11 medium at psyllium concentrations higher than 15 g L^{-1} (as gelling agent) might be due to higher viscosity of the psyllium at these concentrations, which provided less surface area and possible nutrient deficiency for the development of the respective microalgae. The results are partially in line with Sahay (1999), who had very similar observations in microbial culture. He found that a minimum of 5% psyllium gelled medium and 20 min. uv treated 4% psyllium + agar gelled medium/ oven sterilized (120°C for 1 h) ground psyllium husk mixed with autoclaved medium at 50°C with gentle shaking under aseptic conditions just before pouring

yielded a gel as good as given by agar gelled medium for the culture of *Aspergillus niger, Penicillium corymbiferum, Alternaria solani* fungi (eukaryotes) and *E. coli* bacteria (prokaryote). However, we found that this practice (5% psyllium gelled medium and 20 min. uv treated 4% psyllium + agar gelled medium/oven sterilized (120^{o}C for 1 h) ground psyllium husk mixed with autoclaved medium at 50^{o}C) deteriorated the gelling quality of psyllium (isubgol) due to excessive drying of psyllium. Neither *C. limneticus* nor *S. quadricauda* cells could be cultured on this gel successfully (results not given).

Different responses of prokaryotic *C. limneticus* and eukaryotic *S. quadricauda* microalgae should be accepted as normal since the growth requirements of both vary. Similarly Babbar and Jain (1998) recorded statistically similar results on agar or psyllium gelled media for in vitro seed germination, shoot formation and rooting of *Syzygium cuminii* and anther culture of *Datura innoxia* (Eukaryotes). However, partial multiplication of one (prokaryotic microalgae - *C. limneticus*) and satisfactory multiplication of the other (eukaryotic green microalgae - *S. quadricauda*) on psyllium gelled media, will remain within the realm of speculation till the exact cause(s) is/are identified. As for as the cost is concerned, psyllium is very cheap (10 Euro per kg) compared to agar [Sigma Aldrich (A5306) with price of 646 Euros per kg], which makes psyllium highly cost-effective gelling agent. Moreover, psyllium like agar is biodegradable and do not pose any threat to the environment when dispensed-off after use.

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