Appl Microbiol Biotechnol (1989) 31:79–83

Applied and Microbiology Biotechnology

Alginate-based solid media for plant tissue culture

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Summary. A new method for solid medium plant tissue culture based on in situ gelation of alginate is proposed as an alternative to agar-based media. In situ gelation by the use of dispersed $CaCO_3$ and the slowly hydrolysing acid glucono-δ-lactone (GDL) was the basis for the use of alginate as a gelling agent. Inexpensive alginate-based media can be made in a wide range of pH values. Biological tests of these gels, concerning sterile seed growth and microcalli plating of *Brassica napus* (cv. Topas) and biomass production of *Panax ginseng* callus, showed results equal to those achieved with agar-based gels.

Introduction

Rapid development within the area of biotechnology has created new and increasing demands for gelling agents. Solid medium culture within all fields of biotechnology has so far mainly been based on agar/agarose systems, but recently some new gelling agents have been introduced (e.g. Plantgar™). Another alternative to agar-based solid medium culture could be gels made of alginate.

Alginates are co-polymers containing α-L-guluronic acid (G) and β-D-mannuronic acid (M) distributed in homopolymeric blocks with inserts of sequences containing both monomers. Solutions of Na-alginate form gels when divalent or multivalent cations are introduced, Ca^{2+} ions are most frequently used. The affinity for Ca^{2+} and the ability to form strong gels are correlated with the total content of L-guluronic acid (Smidsrød 1972; Smidsrød and Haug 1972) and to the length of the G-blocks (Skjåk-Bræk et al. 1986). The chemical composition of alginates varies with the source of origin (algal species and tissue) and the season of harvest (Haug 1964).

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Entrapment of single plant cells and protoplasts in Ca-alginate gel beads is now an established technique with many applications (Brodelius et al. 1979; Draget et al. 1988). Such beads are generally strongly inhomogeneous in structure, with maximum alginate concentration and gel strength at the surface (Skjåk-Bræk et al. 1989). So far, only Mbanaso and Roscoe (1982) have reported on alginate as a gelling agent for solid medium culture. Their method was based on a balanced 50/50 mixing of stock solutions of alginate and Ca^{2+}. Similar procedures may be applied at high ionic strength, such as in sea water-based media for marine algal cells and tissue (Østgaard et al. 1987). At lower ionic levels, however, gel formation tends to start before the Ca^{2+} ions are evenly distributed, leading to irregular gel lumps of variable density. A general method with practical applicability should be based on gel formation by a more controlled release of calcium ions.

In industry several methods are in use for in situ gelation of alginate. Solutions of sodium alginate can be mixed with a slightly soluble calcium salt, such as $CaSO_4$, and small amounts of complexing agents such as phosphates added to obtain a slow release and steady supply of calcium ions into the solution. Another method (Toft 1982) consists of mixing sodium alginate with a soluble calcium salt of a complexing agent such as ethylenediamine tetraacetate (EDTA) and using the slowly hydrolysing glucono-δ-lactone (GDL) to lower the pH and release the complexed calcium ions into the solution. The problems with the last method for the present type of usage are related to the low pH (4.7) necessary to obtain liberation of Ca^{2+}, and the ambiguous influence of EDTA on plant tissue culture (Dalton et al. 1983). The idea in the present paper is to combine these two approaches, i.e. to use insoluble $CaCO_3$ both

as a source of calcium ions and as a proton acceptor, and GDL to lower the pH to produce the soluble $Ca(HCO_3)_2$ even at neutral pH values.

Materials and methods

Alginate-based gelling system. Dry $CaCO_3$ (15 m*M*, Merck, Darmstadt, FRG) was dispersed in the appropriate liquid (distilled water or cultivation media with low calcium content) and 1% (w/v) dry alginate, LF 10/60 from Protan A/S (Drammen, Norway), was added. This dispersion was autoclaved at 120° C for 25 min and cooled to room temperature giving a sterile solution of dissolved alginate. Glucono-δ-lactone (GDL, Sigma, St. Louis, USA) was dissolved in a small volume (5–10 ml) of distilled water and sterile filtered into the alginate/$CaCO_3$ suspension to initiate gelation. It is of critical importance that this GDL solution is freshly made because fully hydrolysed lactone will initiate instant and uncontrolled gelling. Unless otherwise stated, all gels were made in sterile 9-cm petri dishes and all alginate gels were made of 15 m*M* $CaCO_3$ and 32 m*M* GDL. Agar (Difco Bactoagar, Detroit, USA) was used in agar-based control gels.

Physico-chemical properties. To identify the final pH in this gelling system, 1% alginate solutions in water containing 12.5 and 15 m*M* $CaCO_3$ were supplied with 5–50 m*M* GDL. The mixed solutions were left overnight, and pH was measured by extensively homogenising a sample of each gel with a glass rod in a tube before the pH electrode was introduced.

To study gel strength dependence on polymer concentration, gels of both alginate and agar were made in water with polymer concentrations ranging from 0.50% to 2.00% (w/v). Gel strength was measured as the force necessary to compress a 8 mm-thick gel plate by 2 mm with a Stevens LFRA Texture Analyser (C. Sterens & Son, St. Albans, UK).

Biological properties. Seeds of *Brassica napus,* cv. Topas, were sterilized for 45 min with hypochlorite followed by extensive washing with sterile water. The seeds were placed on gels containing 2 g/l sucrose. Agar gels also contained 3 m*M* $CaCl_2$ and alginate gels were made of 15 m*M* $CaCO_3$ and 30 m*M* GDL. Germination efficiency was estimated by counting viable hypocotyls after 5 days. As a further test of the general condition of the hypocotyls, protoplasts were isolated according to the method of Glimelius (1984). Protoplast yield obtained from agar and alginate gels was measured as the total amount of viable protoplasts isolated from hypocotyls from a fixed number of petri dishes.

Protoplasts of *B. napus,* cv. Topas, were grown in A-medium (Kao and Michayluk 1981) containing 0.4 *M* glucose, 1.0 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 0.5 mg/l 6-benzylaminopurine (6-BAP) and 0.1 mg/l 1-naphthalene acetic acid (NAA). They were grown for 4 weeks to allow formation of a microcallus. Alginate- and agar-based solid A-medium (with 0.1 *M* sucrose, 1.0 mg/l 6-BAP, 0.1 mg/l NAA and without calcium in alginate) were prepared by sterile filtering a solution of double concentrated medium and adding an equal volume of autoclaved double concentrated polymer solution. An equal number of microcalli from the same batch of protoplast isolation was plated both on agar and alginate-based solid medium. Plating efficiency was quantified as the number of microcalli growing to viable calli within 30 days on solid medium. Culturing conditions were 40 W/m^2 light intensity and a day/night regime of 16 h light/8 h dark at 25° C.

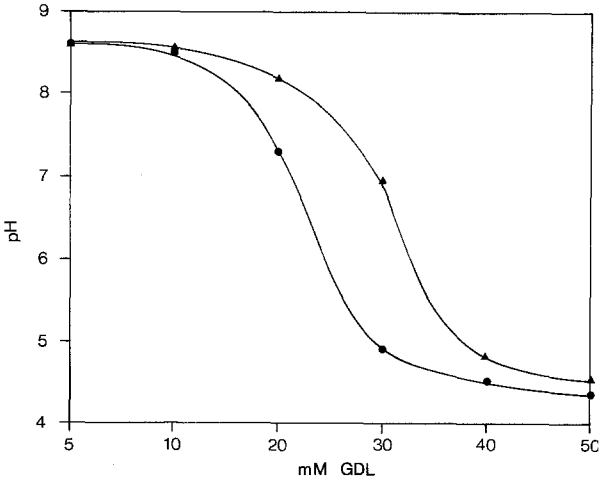

Fig. 1. Final pH in the described alginate gelling system with varying amounts of glucono-δ-lactone (GDL) and $CaCO_3$: ● = 12.5 m*M*; ▲ = 15 m*M* $CaCO_3$

From an already established callus culture of *Panax ginseng,* 1.0 g of callus tissue was placed on alginate- and agar-based Gamborg B5 medium (Gamborg et al. 1968) (freeze dried from Flow Laboratories, Irvine, UK) containing 20 g/l sucrose, 1.0 mg/l NAA and 1.0 mg/l 2,4-D. These gels were made in sterile autoclavable glass tubes with plastic lids. Gels with different polymer concentrations and $CaCO_3$ content were also screened for growth effects. Biomass growth was defined as the relative increase in fresh weight of the callus 8 weeks after inoculation. Culturing conditions were the same as for the *Brassica* microcallus.

Results and discussion

Physico-chemical properties

Depending on the amount of added GDL in the described system, gelling took place within 15 min to 24 h. Figure 1 shows the final pH in the alginate gelling system with varying amounts of GDL. The pH changed according to an acid/base titration curve; i. e. equivalent amounts of $CaCO_3$ (a 2-proton acceptor) and GDL (a 1-proton donor) gave neutral gels. Plant media are normally in the pH range 5.8–6.0, corresponding to 32–34 m*M* GDL when 15 m*M* $CaCO_3$ was used.

Gel strength as a function of polymer concentration is presented in Fig. 2. Agar gels increased in strength with increasing polymer content, while alginate gels at a given $CaCO_3$ concentration had maximum gel strength at polymer concentrations corresponding to equivalent amounts of uronic acids with respect to potentially free Ca^{2+}. This calculation is based on uronic acid monomers with a molecular mass of 200 daltons and one negatively charged carboxy group per monomer.

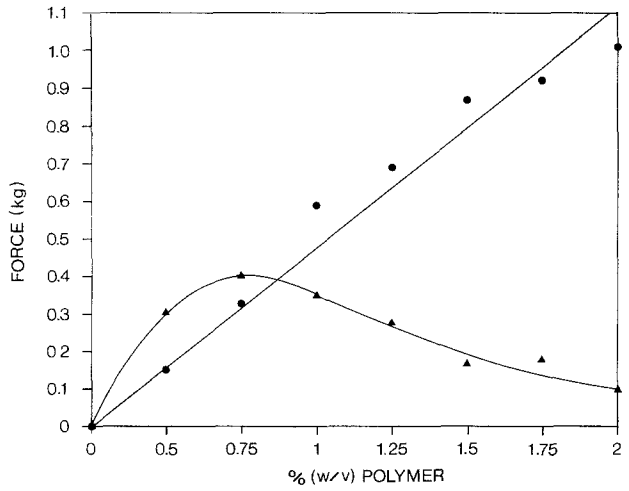

Fig. 2. Gel strength as a function of polymer concentration of agar (●) and alginate (▲). Alginate gels were made with 15 m*M* $CaCO_3$ and 32 m*M* GDL

This gives rise to the somewhat unusual conclusion that at a given concentration of $CaCO_3$, a further addition of polymer results in a weaker gel. The maximum gel strength was shifted to a higher polymer concentration by adding more $CaCO_3$, but this led to water liberation and gel shrinking (syneresis). However, results (not included) from a corresponding series with CaEDTA as Ca donor supports the conclusion that maximum gel strength is reached when the internal content of Ca^{2+} is large enough to bind to all uronic acids residues. The final recipe became 1% (w/v) alginate, 15 m*M* $CaCO_3$ and 32 m*M* GDL.

In addition to Protanal LF 10/60, which is a high-G alginate (Grasdalen et al. 1979), other alginates with a lower content of guluronic acid, such as *Macrocystis* alginate (Sigma), have successfully been used. In tests with different batches of alginate, aggregation was observed a few times with some batches of high-G alginates, probably related to the presence of $CaCO_3$ during autoclaving. Some preliminary testing of the alginate batches may therefore be necessary before use. Alginate-based gels were generally smoother and less turbid than agar-based gels. This may be an important advantage for inverted microscopical inspection of the dishes.

Sedimentation of carbonate before it is completely hydrolysed may give inhomogeneous gels with a dense lower part and a sloppy upper part when making gels deeper than approx. 2 cm. This problem can partly be compensated for by using high viscosity alginate which prevents carbonate sedimentation. Sedimentation is, however, negligible with moderate amounts of $CaCO_3$ and shallow gels.

The accessibility of nutrient ions in alginate gels may be affected by the charged polymer. Some calcium will be tightly bound by the uronic acid residues involved in the junctions of the gel binding zones, corresponding to <5 m*M* Ca^{2+} for a 1% alginate solution. The rest of the ions in the gel are free or reversibly attached to non-structural uronic acid residues. They should therefore be easily available to the growing plant tissue culture. The alginate polymer itself is originally non-toxic, less toxic than sulphate-containing agar.

Biological properties

Table 1 shows typical data concerning germination of sterile seeds of *B. napus*, cv. Topas, and protoplast yield from the resulting hypocotyls. The plating efficiency of 4-week-old microcallus of *B. napus* is presented in Table 2, and the relative biomass growth of established callus of *P. ginseng* after 8 weeks is shown in Table 3.

Table 4 shows biomass growth of *P. ginseng* on alginate-based medium gels with different polymer- and $CaCO_3$-contents. Highest growth was observed at the presumed optimal concentrations given in the final recipe, i.e. 1% alginate and 15 m*M* $CaCO_3$.

Table 1. Sterile seed germination of *Brassica napus* (cv. Topas) and protoplast yield from resulting hypocotyls for corresponding series of 1% agar- and alginate-based gels (±SD)

Experiment	% Germination		Protoplast yield	
	Agar	Alginate	Agar	Alginate
1	97.5 (±3.1)	92.8 (±4.6)	2.62×10^5	1.46×10^5
2	79.0 (±5.0)	83.7 (±3.0)	4.35×10^5	7.05×10^5
3	91.3 (±7.8)	86.4 (±9.8)	3.65×10^5	3.35×10^5

Table 2. Plating efficiency of microcalli from *B. napus* (cv. Topas) on 1% agar- and alginate-based A-medium gels

Experiment	Agar	Alginate
1	18 (± 2)	14 (±11)
2	48 (±31)	20 (± 5)
3	74 (±20)	135 (±43)
4	8 (± 2)	25 (±10)
5	90 (±58)	82 (±56)

The figures indicate number of microcalli continuing growth after 30 days on solid medium (±SD)

Table 3. Biomass increase of callus culture of *Panax ginseng* on 1% agar- and alginate-based Gamborg B5 medium gels

Experiment	Agar	Alginate
1	2.72 (±0.84)	1.95 (±0.36)
2	2.67 (±0.62)	3.28 (±0.78)
3	2.31 (±0.47)	1.42 (±0.63)

Growth was measured as the relative increase in fresh weight in 8 weeks (±SD)

Table 4. Biomass increase of *P. ginseng* on alginate-based Gamborg B5 medium gels as a function of A: alginate concentration (all systems with 15 m*M* $CaCO_3$ and 32 m*M* glucono-δ-lactone (GDL) and B: $CaCO_3$ content (1% alginate and 22, 32 and 42 m*M* GDL respectively)

A:	1.0%	1.5%	2.0%
	1.55 (±0.36)	1.38 (±0.54)	0.99 (±0.31)
B:	10 m*M* $CaCO_3$	15 m*M* $CaCO_3$	20 m*M* $CaCO_3$
	2.24 (±0.54)	3.28 (±0.78)	1.37 (±0.32)

Growth for both systems was measured according to Table 3 (±SD)

Variations in the biological data from independent experiments, a general experience when dealing with such plant tissue culture systems, is mainly due to variations in the handling and quality of the biological material from time to time. Tables 1 to 3 show high biological activity on both agar and alginate in the same experiment, and low activity in the same experiment. The data sets obtained for agar and alginate (Tables 1-3) can therefore not be treated as statistically independent. Statistical comparison of biological activities on agar- and alginate-based media is therefore carried out by examining a ratio (α = results obtained on alginate/results obtained on agar) calculated from data obtained in the same experiment. The main question in this investigation will then simply be whether the mean value of α is significantly different from 1.0 or not. As a rough approximation, one can assume that the variations in α in independent experiments are normally distributed. Statistical significance can then be estimated using Student's *t*-test. Table 5 summarizes statistical calculations of results from all biological systems tested. It is concluded that these alginate-based gels show no significant inferiority when compared to agar-based control gels.

Finally, we would like to stress that alginate itself is not a direct substitute for agar, but rather an alternative basis for a new gelation method. This completely different gel formation may be advantageous in cases where high temperatures must be avoided. Alginate gels may also be easily redissolved without damaging embedded tissue (Brodelius 1984; Draget et al. 1988). Most important, alginate gels can be made considerably more cheaply than agar-based gels. In any case, this gelling system should be tested out on a small scale using the actual biological system before it is adopted, to ensure that biological activity is satisfactorily retained.

Acknowledgements. We would like to thank Heidi Bente Hoel Draget for technical assistance. This work has been supported by Protan A/S, Drammen, Norway, and the Norwegian Research Council for Science and Technology (NTNF).

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Table 5. Statistical treatment of results presented in Tables 1-3

Biological system	No. of independent experiments	Alginate/agar (±s.e.m.)	Statistical significance
% Germination of *B. napus*	3	0.986 (±0.03)	n.d.
Protoplast yield of *B. napus*	3	1.032 (±0.31)	n.d.
Microcalli plating of *B. napus*	5	1.411 (±0.63)	n.d.
Biomass growth of *P. ginseng*	3	0.853 (±0.19)	n.d.

The ratio of results obtained on alginate/agar was tested for significant deviation from 1.0 by Student's *t*-test ($P<0.05$) in each biological system; n.d., not different; s.e.m., standard error of the mean

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Received 23 November 1988/Accepted 17 January 1989