Characterization of Agarose Product from Agar Using DMSO

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Agar was extracted from *Gelidium amansii*, which was harvested at the shores of Jeju Island in South Korea. As a unique solvent, the ability of dimethyl sulfoxide (DMSO) was used to separate agarose from agar by removing agaropectine and quality of the resultant agarose was characterized for chromatography purposes. Agar sample was agitated by motor-driven stirrer with DMSO in a water bath (at 70°C for 2 h) and centrifuged (3,000 rpm for 20 min). Resultant upper agarose layer was gelled, washed, dried and milled. The quality of agarose was evaluated by the analysis of proximate chemical composition, sulfate content, gelling strength and DNA migration. In this study, the separated agarose showed low sulfate amount (0.28%) and showed high gel strength (1190 $g \cdot cm^{-2}$). The resolution power and the ligase activities gave clear picture about the suitability of the present agarose for practical purposes.

Key Words: agar, agaropectine, agarose, DMSO, DNA migration, gel strength

INTRODUCTION

Gels are widely used for separation of biological molecules by techniques such as gel filtration, ion-exchange chromatography, as well as for immobilization of biocatalysts (Armisen 1997; Fuji *et al.* 2000; Millan *et al.* 2002). Agarose, one of the neutral gelling substances is widely used not only in the field of biotechnology but also in many industries.

Agarose is a linear, neutral marine polysaccharide consisting of alternating (1,3)-linked-β-D-galactopyranose and 1,4-linked-3, 6-anhydro-α-L-galactopyranose that is separated from agar by removing agaropectine. The polymer exists as a random coil in dilute or hot solutions but undergoes conformational transitions to form ordered helices in the solid state (Guiseley 1970). The large pore size, low electroendosmosis and the strength of the matrix of agarose have advantages over other media such as polyacrylamide in many applications. Due to its unique nature, agarose is applied to the biotechnology area as a key element in powerful techniques such as gene mapping and for special gel electrophoresis methods where a non-ionic solid is required (Abbot 1990).

With the increment of biotechnological applications, agarose has become the highest priced seaweed product sharing 0.2% of the phycocolloid market (Critchley 1997). Agarose is manufactured by removing the ionic fractions of agar under highly controlled conditions designed to minimize variation (Radmer 1996). The traditional separation of agarose mainly depends on DEAE-cellulose, a weaker anion exchanger. The methods involved in separation of agarose are highly complex and require intensive separation techniques in order to minimize lot-to-lot variation. The unit value of some of these products can be impressive, ranging beyond 25,000 $/kg (Radmer 1996). The possibility of agarose production from agar by extraction, batchwise ion exchange has been investigated. The purification efficiency and adsorption capacity of agarose obtained from the above methods are highly controversial. Therefore, finding alternative simple and productive method for good quality agarose is highly desired.

The aim of this study is to investigate preparation of high quality agarose from agar by simple separation techniques. Dimethyl sulfoxide (DMSO), a unique solvent, was used to separate agarose from agar by removing agaropectine and characterizations of the agarose prepared in this method were examined.

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MATERIALS AND METHODS

Materials

Marine alga, *Gelidium amansii*, was collected close to the shores of Jeju Island in South Korea. Samples were cleaned with freshwater and freeze dried at -20°C for further experiments. Dimethyl sulfoxide (DMSO), HCl, K_2SO_4, and other chemicals were purchased from Sigma Chemicals Co. (St Louis, Mo), (USA). Commercial agarose-LE was obtained from Promega Corporation (USA). DNA ladder marker and T4 DNA ligase were purchased from New England Bio-lab (UK) and Takara Bio Inc (Japan), respectively.

Methods

Agar extraction: Agar extraction was performed as described by Mollet *et al.* (1998). Briefly, dried seaweed sample was autoclaved for 1hr (at 120°C) in distilled water at pH 7.5. The insoluble particles of the resulted aqueous solution were removed using membrane filtration (500 μm to 1.7 μm). The filtrate was allowed cooled down at room temperature, frozen overnight and thawed at 20°C. Thereafter, sample was subjected to centrifugation (5000 × g, 15 min) in order to remove water and to flocculate carbohydrates, and then flocculated carbohydrates mixture was dried at 4°C for 12 h with ethanol 95% (v/v). The dried sample was dissolved in distilled water at 100°C and finally the mixture was dialyzed against water at 4°C for 2 days. Freeze dried agar sample was ground and used for agarose preparation.

Agarose extraction: Agar was cautiously added into DMSO to be 1, 3, 5 and 7 % [w/v] agar solution under stirring at 70°C. After 2 h stirring, the agar dissolved in DMSO was centrifuged (356 × g, 20 min at 4°C) and obtained a sediment, demarcated supernatant that is in general in the form of light yellow colored stiff gel. The stiff gel was separated and dissolved with same amount of distilled water and allowed to make a gel at room temperature. Finally, the obtained agarose gel was broken into small pieces and washed several times with distilled water in order to remove DMSO remained. Excess water was removed by filtering in Buchner funnel, and then the sample was subjected to freeze-drying. Finally, milled agarose samples were obtained and used for further experiments.

Proximate chemical composition of agarose: Proximate chemical composition of the agarose sample was determined by the AOAC (1990).

Gel strength: The agarose/agar gel strength was followed by the modified method as described by Mollet *et al.* (1998). The gel strength was measured as the force necessary for a 1 cm^2 circular plunger to penetrate 3 cm thick slandered agar/agarose gel (1% [w/v]) at room temperature.

Sulfate content: Sulfate content was measured according to the modified method of Mollet *et al.* (1998). Agar and agarose samples were hydrolyzed with 2N HCl (for 2 h at 100°C) in microvials washed with nitric acid, then the sample was evaporated and the sulfate was suspended using purified distilled water. The suspension was filtrated using Whatman filter paper (0.22 μm) and sulfates were purified by HPLC western anion exchange column (4.6 mm × 250 mm, Alltech). The sulfates were eluted using potassium hydrogen phosphate (1 ml/min) and the content was determined using conductometer. K_2SO_4 solution (10 to 200 μg·ml^{-1}) was used as a reference.

Mineral analysis: In measuring mineral compositions of agarose samples, one gram of sample was heated at 600°C in a muffle furnace for 24 h to remove organic constituents and added into a test tube containing a mixture of 4 ml of diluted HCl solution (conc. HCl : DW = 0.5 : 3.5) and 10 ml DW, and then dissolved in a water bath with increased temperatures. The dissolved sample solution was volumerized to 100 ml and applied into ion chromatography (Dionex 600 Ion Chromatography, USA). Analytic method of ion chromatography was as follows: column, IonPac CS-12A; eluent, 22 mN sulfuric acid; flow rate, 1.0 ml/min; injection volume, 10 μl; detector, Suppressed conductivity ASRS-Ultra 4-mm; analytic time, 20 min/sample.

Resolving properties of agarose: The resolution power of agarose prepared from agar in this study was compared with commercially available agarose (Promega Agarose, LE) by running 1 kb DNA ladder marker (from 500bp to 12kb, Gibco BRL) in 0.9% and 100 bp DNA ladder marker (from 100bp to 2,072bp, Gibco BRL) in 2% gels prepared with the two-agarose types in TAE buffer.

Agarose on ligase activity: *EcoR*1 digested pUC19 plasmid purified from commercial agarose and the prepared agarose in this study using AccuPrep Gel Purification Kit (Bioneer, Korea) were ligated using T4 DNA ligase. The ligation reactions were carried out at 15°C for the given time. The reactions were stopped by incubating at 65°C for 10 min and analyzed on 0.8%
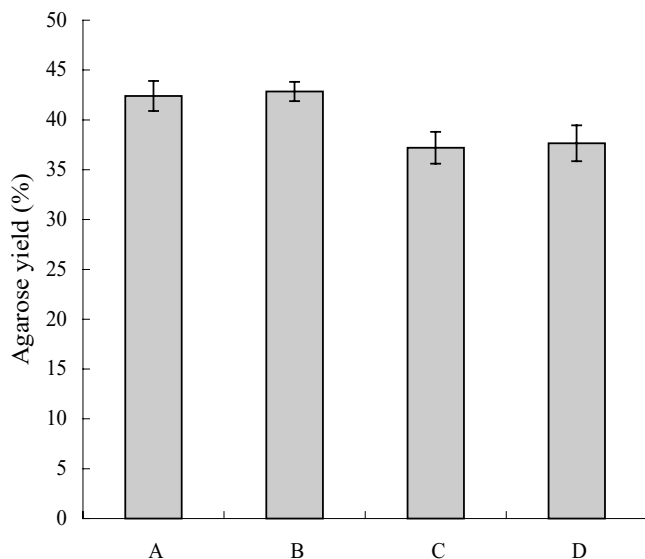
Fig. 1. Agarose yield obtained from different agar concentrations in DMSO (A) Agarose obtained from 1% agar concentration [w/v]; (B) Agarose from 3% [w/v] agar; (C) Agarose from 5% agar; (D) Agarose from 7% [w/v] agar. Yield was expressed as a percentage of dry weight.

Fig. 2. Agarose gel strength ($g \cdot cm^{-2}$) obtained from different agar concentrations in DMSO. (Agar) Parent agar sample; (A) Agarose obtained from 1% agar concentration [w/v]; (B) Agarose obtained from 3% [w/v] agar; (C) Agarose obtained from 5% [w/v] agar; (D) Agarose obtained from 7% [w/v] agar. Gel strength was measured with 1% agarose gel.

agarose gels with control. The same protocols were performed by replacing *EcoR*1 with *Sma*1.

RESULTS AND DISCUSSION

Agarose yield

The extracted agarose yield is shown in Fig. 1. For the treatment with 1% and 3% agar in DMSO (w/v) yielded almost same agarose amount (42%), while 36% agarose yield was obtained from the samples treated with 5 and 7% agar in DMSO (w/v). According to the yield results of this experiment, when increase agar concentration the agarose yield become low, this may be due to inappropriate amount of DMSO to extract agarose from agar. Furthermore, increased amount of agar make high sticky solution after agitation with DMSO, this makes purification difficulty after centrifugation. Therefore despite high concentration of agar, low concentration agar in DMSO gains high yield. Do and Oh (1999) extracted 37.8% agarose yield from agar by using polyethylene glycol. Therefore, present technique gain quite successful yield than polyethylene glycol treated agar. Also, after one cycle of agarose purification it can be re-extracted up to a certain level (data not presented), therefore, agarose yield can be increased with the manipulation of favorable techniques. Meanwhile present agarose preparation technique does not cause
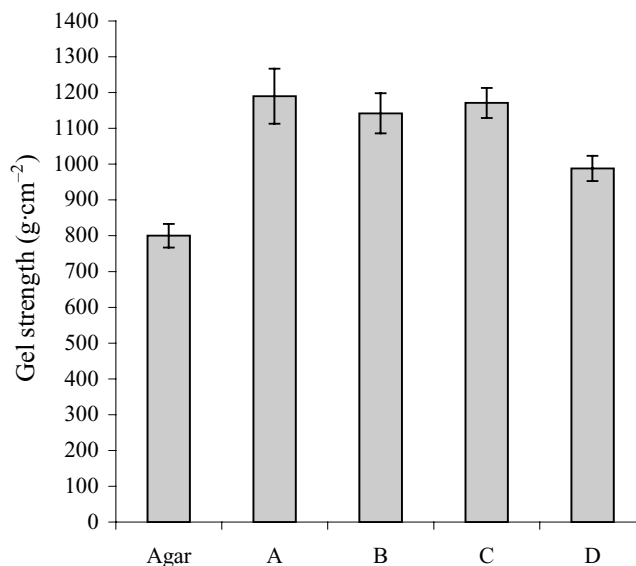
any purification difficulties. Moreover, the resultant agarose was rapidly dissolved in hot water and showed the same texture/colour as commercial agarose. These results may be attributed to the unique qualities of DMSO.

Proximate chemical composition of agarose

It is noteworthy to mention that present technique is useful to make agarose with low ash content (Table 1). Pelegrin and Robledo (1997) observed a similar pattern between ash content and sulfur content, with the agreement of above the finding, which was shown in Table 1 and Fig. 3. Agarose (A) and (B) samples showed 1.06 and 1.21% ash content, respectively. When we compared these results with the ash content (1.28%) of agarose extracted by cetylpyidinium chloride (CPC) (Do and Oh 1999), present technique reduce ash content more effectively than the CPC treatment method. As well as, larger proportion of the agarose was constituted with carbohydrate rather than protein and fat. We can assume this may be due to high accumulation of 3, 6 anhydrogalactose which regards as the main compound of agarose.

Gel Strength

One of the most important factors contributing to the

Table 1. Proximate chemical composition of agaroses

Compounds	Agaroses				
	Agar	A	B	C	D
Moisture	12.70	9.01	8.05	7.98	6.20
Crude Protein	0.52	0.41	0.43	0.43	0.48
Crude lipids	1.20	0.60	0.60	0.60	0.80
Crude ash	1.70	1.06	1.21	1.50	1.50
Crude carbohydrate	83.88	88.92	89.71	89.49	91.02

(Agar) Parent agar sample; (A) Agarose obtained from 1% [w/v] agar in DMSO; (B) Agarose obtained from 3% [w/v] agar; (C) Agarose obtained from 5% [w/v] agar; (D) Agarose obtained from 7% [w/v] agar.

success of agarose as an anticonvection medium is its ability to exhibit high gel strength at low concentrations. The gel strength of the agarose extracted in present study was shown in Fig. 2. According to the results, all agarose samples (at 1% agarose concentration) exhibited good gel strengths (988-1190 $g \cdot cm^{-2}$) and were in the range that require for commercial agarose (>800 $g \cdot cm^{-2}$) or other agarocolloids (Selby and Whistler 1993). The highest gel strength (1190 $g \cdot cm^{-2}$) was obtained from agarose (A) sample while the lowest gel strength (988 $g \cdot cm^{-2}$) was recorded from sample (D) agarose (7% agar [w/v]). For agarose (B) and (C) the gel strengths were between 1142~1171 $g \cdot cm^{-2}$. Therefore it is obvious that the gel strength of agarose (A), (B) and (C) were far higher than that of parent agar sample. In the application process, agarose gel strength is very important; therefore, conditions involved in the agarose preparation must be carefully controlled. The present study, which is more simple and convenient than the traditional methods, produces high gel strength agarose with less effort. The low gel strength attributed with the agarose (D) may be due to inappropriate amount of DMSO. This may be caused by insufficient transformation and conformation of the substitute groups in the agar polymer. DMSO amount treated in 1, 3 and 5% [w/v] agar concentrations induced preparation of good gel strength agaroses.

Sulfate Content

Pelegrin and Robledo (1997) explained that there is an inverse relationship between gelation and sulfate content of agar and agarose. In this experiment, the amount of sulfate contained in different agarose samples was estimated (Fig. 3). According to the results, all the samples constituted with almost similar sulfate content (0.28 to 0.29%) and these sulfate contents were two times lower than that of parent material (0.55%). This may be
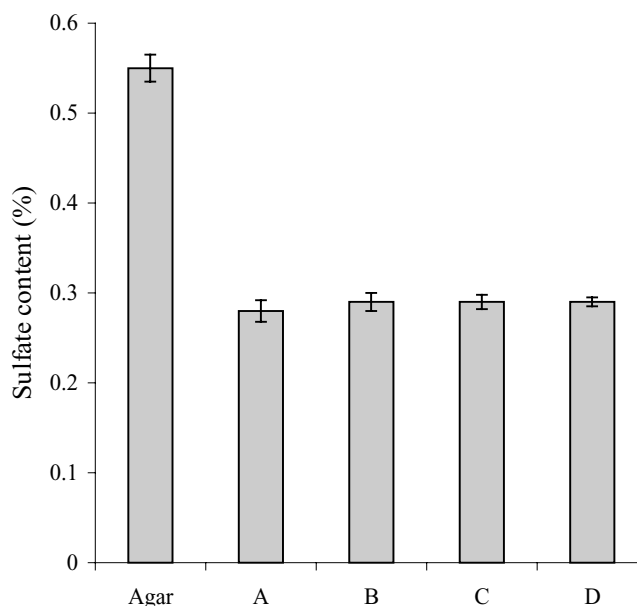

Fig. 3. The sulfate content of agarose samples, (A) Agarose obtained from 1% [w/v] agar concentration in DMSO; (B) Agarose obtained from 3% [w/v] agar; (C) Agarose obtained from 5% [w/v] agar; (D) Agarose obtained from 7% [w/v] agar.

due to incomplete purification. It is aware that all sulfur in agar is probably not in the form of half esters. Double-ester sulfates are also conceivable. Neutral molecule containing such sulfur will not be precipitated completely treating with DMSO followed centrifugation therefore, may contaminate the agarose. Of course, the presence of such non-ionogenic sulfur and sulfur-containing groups in an anticonvection agent for electrophoresis does not involve with any disadvantage (Hjerten 1962), because they do not contribute to electroendosmosis and adsorption. With the agreement of above research findings, all agarose samples in the present experiment showed remarkable gel strength, which is desirable for commercial purposes. Do and Oh (1999) separated agarose from agar using chitosan, cetylpyridinium chloride (CPC) and polyethylene glycol (PEG), and their respective sulfate contents were 0.72, 0.38 and 0.32%, respectively. On the other hand, Hjerten (1971) suggested that polyethylene glycol could precipitate agaropectin easily and separate agarose from agar, but pointed out a fault that it is apparently difficult to remove completely the excess of polyethylene glycol completely. In the present method, however, it is observed that DMSO could effectively remove sulfated agaropectine from agar and there was no difficulty in removing DMSO for further purification.

Table 2. Mineral composition of agaroses.

Agarose	Mineral Composition (ppm)				
	Li^+	Ca^{+2}	Mg^{+2}	K^+	Na^+
Agar	49.27	180.20	31.44	102.41	321.11
Agarose (A)	23.97[a]	54.78	20.63	76.87	151.86
Agarose (B)	20.89	56.32	28.69	75.28	144.69
Agarose (C)	07.15	84.09	41.15	35.37	82.10
Agarose (D)	09.34	151.01	76.62	46.60	82.50

[a] Mineral composition expressed as a percentage of dry weight. (Agar) Parent agar sample; (A) Agarose obtained from 1% [w/v] agar in DMSO; (B) Agarose obtained from 3% [w/v] agar; (C) Agarose obtained from 5% [w/v] agar; (D) Agarose obtained from 7% [w/v] agar.
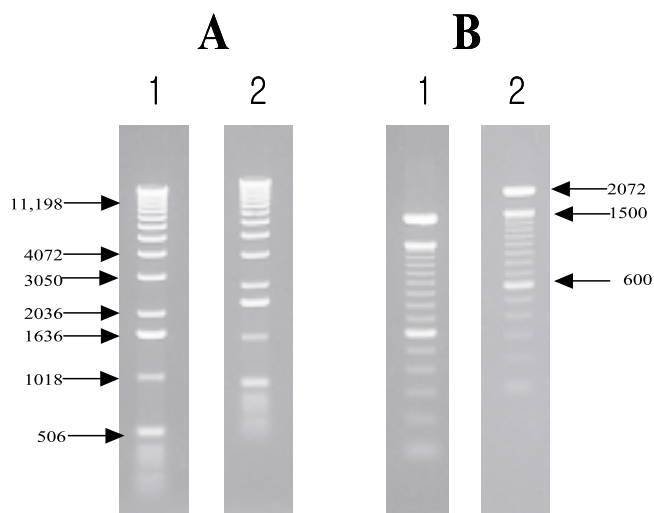

Fig. 4. 1 kb DNA ladder marker (from 500 bp to 12 kb) was fractionated in 0.9% agarose gels prepared from commercial agarose (lane A1, promega LE) and agarose purified in this study (lane A2). 100 bp DNA ladder marker (from 100 bp to 2,072 bp) was fractionated in 2% agarose gels made from commercial agarose (lane B1) and agarose prepared in this study (lane B2).

Mineral Composition

As the results reported by Derbyshire *et al.* (2001) the gelling behavior of agarose is inversely influenced by relatively high concentration of cations. Moreover, small polarizing cations tend to reduce ordering temperature of agarose. In the present experiment, the cation composition was highly varied among the agarose samples (Table 2). The highest lithium concentration (23.97 ppm) was recorded from agarose (A) sample, while the lowest concentration (7.15 ppm) was recorded from agarose (C) sample. However, the lithium ion is not effective in reducing the net hydrogen bonding in the solvent, therefore, it is less/not effective as a temperature depressor in making agarose. As the polarizing power of cation increases, its capacity to change normal cluster structure of water is increased. Therefore, with a high concentration of cations like Ca^{2+} will be more compatible with water structure and affect in melting temperature of agarose. In the present study, with the increment of parent agar concentration (from 1-7% agar [w/v] in DMSO), the Ca^{2+} concentration increased simultaneously from 54.78 to 151.01 ppm. It has been shown from molecular dynamic simulation (Heinzibger 1985) that water molecules may take octahedral positions around the Mg^{2+}. This arrangement is incompatible with the tetrahedral arrangement of water molecules in a cluster (Derbyshire *et al.* 2001). Therefore, resolution power of agarose reduces the net hydrogen bonding. In this study the Mg^{2+} concentration increased simultaneously with the increment of parent agar concentration (from 20.63 to 76.62 ppm). Mg^{2+} acts as a temperature depressor by disrupting the hydrogen bonding in the solvent, therefore the Mg^{2+} amount in agarose sample is important. Agarose samples prepared

from above technique consisted low Mg^{2+} concentration [especially agarose (A) and (B)], which is not enough to make dissolving problems. The sodium ion and potassium ion concentration in the agarose samples showed similar pattern. Lower parent agar concentration (1 and 3% agar [w/v]) showed higher amount of above two ions.

It is obvious that ion concentration of agarose directly associated with the solubility and ordering temperature. Moreover a higher ion concentration makes problems for DNA resolution. Mineral amount in the present agaroses did not show any dissolving problem or did not affect for DNA resolution, as shown in Fig. 4.

Resolution power of agarose

Agarose should have a good DNA resolution power in order to apply for nucleic acid analytical applications. Extremely good agaroses allow outstanding resolution of bands and retention of full biological activity. In the present study, 1 kb and 100 bp DNA ladder markers were fractionated in 0.9 and 2% agarose gels, respectively, and the banding patterns were compared with commercial agarose (Fig. 4). The results of both reactions (A2 and B2) were almost similar to their control counter part (Fig. 4. A1 and B1), and verified the precise banding pattern without any smearing or high background fluorescence. According to the results, it is
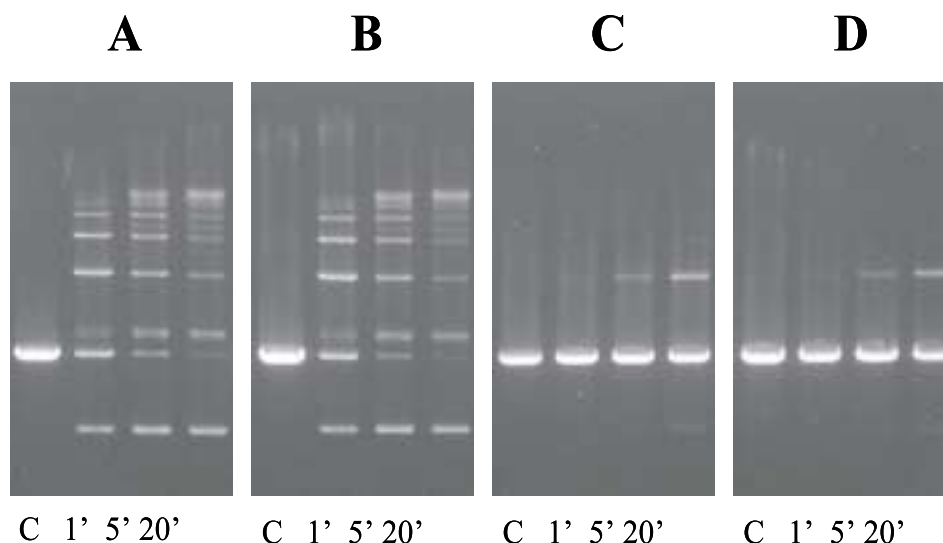
Fig. 5. pUC19 plasmid containing sticky ends purified from agarose gels prepared with commercial agarose (reaction A, Premega) and agarose prepared in this study (reaction B) was ligated using T4 DNA ligase for indicated times. The ligation reactions were fractionated in 0.9% agarose gels with controls in which no ligation was done. In reactions C and D, pUC19 plasmid containing blunt ends was used.

clear that the agarose prepared in this method is suitable for sizing linear double-slandered DNA fragments from 500 bp to 11 kb. The banding pattern in the DNA ladder verified in the present agarose sample was free from contaminants that will cause inhibition or slowdown the DNA migration. These unsmeared, distinct bands make it possible to determine the precise molecular weight and lead to quick and correct nucleic acid analysis, which is essential for practical works.

Effect of agarose on ligase activity

The plasmid has proven to be a useful tool in molecular biology. Therefore, The isolation and manipulation of plasmid DNA has become a routine and essential part of molecular biology. Agarose gel electrophoresis can be employed to check the progression of restriction enzyme digestion of plasmids and thus useful in determining the yield and purity of DNA (Fig. 5). In this study, linearized pUC 19 plasmid containing sticky ends was prepared from a commercial agarose gel and ligated using T_4 DNA ligase (Fig. 5, reaction A and B). The results were compared with the agarose prepared in this study. For the plasmids containing sticky end purification, reactions showed clear, separate extinction-banding pattern (as observed from commercial agarose sample). This result implies that agarose prepared newly in this study does not make any physical or chemical disturbances on linear sticky-end plasmid separation. A similar experiment with

blunt-end ligation (Fig. 5, reaction C and D) also indicated the suitability of the agarose for the separation of DNA. In this experiment, reaction A results show similar banding pattern with reaction B results likewise, reaction C blunt-end ligation banding pattern also totally tally with that of reaction D results. This gives clear picture about the purity and suitability of the present agarose for practical purposes.

ACKNOWLEDGEMENT

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