Chapter: 6

6.0: Improved Culture Practices

The *in vitro* cultivation of plant tissues is generally carried out in a solid or semi-solid nutrient medium, using gelling agents. Traditionally, agar is used, which is a polysaccharide extracted from seaweeds. The main differences among different agar-products are due to the impurities, their level and composition, which can vary according to manufacturers. Agar has been widely used since it has convenient gelling properties and stability during tissue culture. In all media it is used for *in vitro* culture of plants and microbes, besides it's high costs.

Instead of Agar other substrates of gelling agents like Isubgol, Guargum, Natural gum, Rice flour, Singoda flour, Sabudana, Suji use to standardize protocol for tissue culture medium to reduce cost for economical viable in tissue culture commercial industries.

Gums, such as gelan, produced by bacteria and commercialized under the name of Gel-Gro®, Gerlite® (Kelko, Merck) and Phyta-gel® (Sigma), are polysaccharides that do not contain contaminating materials. Moreover, these products are used in lesser amount perliter than agar, to obtain the same consistency. They are added to the medium at approximately one fourth the concentration of agar. Further more, they appear more transparent. Despite emerging as alternatives to agar, the high cost of these products still limits their use in commercial cultures. These polysaccharides are imported from North America and Europe and therefore this leads to increasing costs for further micropropagation applications.

An alternative for cost reduction is the partial replacement of some of these gelling agents with other polysaccharides. Starch is an inexpensive alternative among studied gelling agents, and its use may reduce the costs of tissue culture. Nevertheless, starch is hydrolyzed by plant amylolytic enzymes during the *in vitro* culture. To circumvent this occurrence, the increase of air exchange

in the bottle and consequently the increase of evaporation of excess water, may reduce this drawback (Pinho 2002).

In early experiments, using maize starch as gelling agent, the growth and differentiation of cultured plant cells from tobacco and carrot have been increased. In a medium solidified with starch, cell dry weight increased more than three times with respect to cells grown in a medium gelled with agar (Henderson and Kinnersley 1988).In India, several tissue culture studies have been conducted using gums and starches derivedfrom tropical species, such as true sago palm (*Metroxylon sagu*) and "isubgol" (*Plantago ovate*), Katira gum, derived from the bark of *Cochlospermum religiosum*, was used as gelling agent inthe culture medium. Guar gum, derived from the endosperm of *Cyamopsis tetragonoloba* and locust bean gumfrom carob (*Ceratonia siliqua*).

A gelling agent developed in Brazil (patent PI9003880-0 FAPESP/UNESP) was tested as analternative to agar in the micropropagation of sweet potato (*Ipomoea batata*). The productconsists of a mixture of starch from seeds of pigeonpea (*Cajanus cajan*) and cassava starch (*Manihot esculenta*) at a ratio of 2:3, being used as a gelling agent in the MS culture mediumat a concentration of 7%. It was observed that this starch mixture increased the fresh weight of cultures when compared to microplants grown on agar. Thus, this starch mixture represents a good alternative for agar replacement in the micropropagation of sweet potato.Moreover, this substitution reduced by over 94% the final cost of the culture medium, demonstrating a high economic validity (Bhattacharya *et al.* 1994).

In current investigation, there are seven substitutes which are used as gelling agents instead of agar. These include,Riceflour (RF), Isubgol (IS), Naturalgum (NG), Sabudana (SD), Suji (SJ), Singoda flour (SN) and Guargum (GG).

6.1: Rice flour

Common Name : Rice (chawal)

Botanical Name *: Oryza sativa*

Family : Poaceae

6.1A: Cultivation: Rice is mainly produced & consumed in Asia region, India ranks second in production after China.

6.1B: Chemical composition: Rice is food but made up of natural chemicals mainly proteins, carbohydrates &lipids protein include around 15 aminoacids, the most abundant being glutamic acid.Rice flour is finely granulated powder made by grinding.

Protein: 6.0-9.0%

Fat: 0.4-1.o%

Crudefiber: 0.3-1.0%

Ash: 0.6-0.8%

6.1C: Physical Characteristics

Appearance : White to creamy powder which is relatively free from specks.

Aroma : Typical rice aroma, free from sour musty.

Flavor : Typical rice flour with no rancid or off flavors.

6.2: Isubgol

Its indigenous natural dietary fiber, the husk mucilage is a clear,colorless gelling agent able to increase in volume upon absorbing water upto 40 times its own weight.

Common name : Isubgol

Botanical name : *Plantago ovate*

Family : Plantaginaceae

6.2A: Cultivation: It's a native of Persia, now grown in the western part of India. Main producing states are Gujarat, Rajasthan, Madhya Pradesh and Haryana. As a cash crop in the Mehsana, Patan & Banaskantha district of north Gujarat. Presently India is largest producer and among above states Gujarat is main hub for production as well as processing.

6.2B:Market: India has largest market share in production, processing & export of psyllium husk(~36%) among all the contributing countries during 2002/2003, India s export was about 25.583 tones of psyllium husk,worth INR 2.5billion & 404 tones of seed,worth INR 2 million. Indian export of isubgol seeds and husk. It is exported worth more than Rs 25 million annually from total production of husk in Gujarat, 75%.

6.2C: Chemical composition: It contains ~15% non polysaccharides & remaining 85% appears to consist of a single polysaccharides comprising D-xylose(~62%), L-arabinose(~20%), L-rhamnose(~9%) and D-galactouronic acid(~9%). Out of two polysaccharide fraction separated from husk; one is soluble in cold water & while another is soluble hot water.

6.3 : Sabudana

Sabudana, a whole-grain starch extracted from the pith found inside the trunk of Sago Palm, scientifically known as *Metroxylon sago*. Sabudana resembles pearl tapioca, which is derived from the cassava plant. Pearl sago's whitish seeds become soft and spongy when soaked in water, and turn translucent when cooked. Sabudana makes up the staple food of the lowland peoples of New Guinea as well as Moluccas.

Common name : Sabudana

Botanical name : *Manihot esculenta* Crantz

Sabudana is mostly produced by Tamilnadu, Karnataka, Andhra pradesh, Gujarat and Maharashtra.

6.3A: Chemical composition: They are a source of pure carbohydrate with very little protein, Vitamin C, calcium and minerals. One hundred grams of dry sago yields 355 calories, including an average of 94 grams of carbohydrate, 0.2 grams of protein, 0.5 grams of dietary fiber, 10 mg of calcium, 1.2 mg of iron, and traces of fat, carotene, thiamine, and ascorbic acid.

6.4 : Natural Gum

Natural gums are polysaccharides of natural origin, capable of causing a large viscosity increase in solution, even at small concentrations. Natural gums can be classified in the food industry they are used as thickening agents, gelling agents, emulsifying agents, and stabilizers. In other industries, they are also used as adhesives, binding agents, crystal inhibitors, clarifying agents, encapsulating agents, flocculating agents, swelling agents, foam stabilizers, etc. Most often these gums are found in the woody elements of plants or in seed coatings.

Natural gums can be classified according to their origin. Natural gums can be obtained from Seaweeds like Gelidium and Gracillaria (Agar, Alginic acid, Sodium alginate), Non-marine botanical resources (Gum Arabic from acacia tree, Gum ghatti from Anogeissus trees, Karaya gum from Sterculia trees) Produced by bacterial fermentation (Gellan gum).

6.5 : Suji

Semolina or Suji is the heart of the durum wheat (hard wheat) kernel. It is high in gluten. The milling process separates the endosperm, which is the nutritious heart of the grain. Semolina is a fine, granular flour made from Durham wheat. Also marketed by the name farina, semolina is used to make pasta, Cream of Wheat cereal, and is the basis for flat breads, cakes and pastries around the world. Like bread flour, Durham has high protein. The protein in wheat, called gluten, creates elasticity when moistened and kneaded. Durham wheat has even more gluten protein that bread flour. It is the extra gluten that allows the pasta to stretch so thinly.

Common name : Semolina (Suji)

Botanical name : Durum wheat

Family : *Nicoletella semolina* Kuhnert *et al.*

6.6 : Guar gum

Guar gum also called guaran, is a galactomanna. It is primarily the ground endosperm of guar beans. The guar seeds are dehusked, milled and screened to obtain the guar gum. It is typically produced as a free-flowing, pale, off-white-colored, coarse to fine ground powder.

Common Name : Guar

Botanical Name : *Cyamopsis tetragonolobus.*

Family : Fabaceae

Genus : Cyamopsis

Part used : seed

6.6A:Chemical composition: Commercial food grade guar gum is reported to contain usually about 80% guaran(a galactomannan),5-6%crude protein,8-15%moisture, 2-5% crude fiber,0.5-0.8%ash, and small amount of lipid composed mainly free and esterified fatty acids.

6.6B: Physical characteristics: Guar Gum is a white to yellowish white powder and is nearly odorless. Fine finished Guar Gum Powder is available in different viscosities and granulometries depending on the desired viscosity development and applications. Guar gum is a cold water soluble polysaccharide, consisting of mannose and galactose units. This ability to hydrate without heating makes it very useful in many industrial and food applications.

Dissolved in cold or hot water, guar gum forms a slime of high viscosity. Guar's viscosity is a function of temperature, time, and concentration. Solutions with different gum concentrations can be used as emulsifiers and stabilizers because they prevent oil droplets from coalescing. Guar gum is also used as suspension stabilizer.

Use of different carbohydrates in combination with agar. These includes Rice flour ,Isubgol, Natural gum, Sabudana, Suji & Guar gum by checking the gelling effect of this things with lower concentration of agar than its standards concentration of agar, The best result of gelling at minimum concentration of these includes;

(a) Rice flour :

Agar - 3gm
Rice flour - 50gm

(b) Isubgol :

Agar - 4gm
Isubgol - 25gm

(c) Natural gum:

Agar - 4gm
Natural gum - 10gm

(d) Sabudana :

Agar - 4gm
Sabudana - 10gm

(e) Suji:

Agar - 4gm
Suji - 10gm

(f) Guar gum:

Agar - 3gm

Guar gum - 25gm

6.7 : Materials and Methods

Gelling agent, sources and gelling ability: The gelling performance of various potential gelling agents was investigated with MS broth as the basal medium. Treatment consisted of rice flour, isubgol, natural gum, sabudana, suji and guar gum. Guargum was obtained from, while remaining agents were procured from the local market. Gelling agents at various concentrations were added to the basal medium and heated for 5-10 minute to get good solution bottles that containing agar only served as control. These different gelling agents in different concentration with different agar concentration dispensed into bottles to determine their gelling ability and this solution was heats for few minutes. After 12 hrs incubation gelling has occurred.

Different gelling agent used in combination with agar in different concentration.

Preparation of MS broth medium: This medium is prepared by using sugar can act solely as a carbon source or as an osmotic or both. Sugar serves as a carbohydrate supply to provide an optimal culture condition for cell. They enter the metabolism pathways and transformation of energy which are required for growth of cell.

The pH of this solution is adjusted to **5.85**. As a gelling agent combination of agar and different polysaccharides like rice flour, isubgol, natural gum, sabudana, suji, guar gum respectively,is used after making this preparation, solution is slightly heated for 5-10 minutes. After boiling this medium, its immediately pour into bottles and labeled accordingly and autoclaved.

Inoculation and Incubation: Inoculation was carried out in a sterile laminar air flow hood chamber, surface sterilization was achieved through spraying 70% alcohol for experiment-

culture of stevia inoculated in a bottle containing MS media with combination of agar with different polysaccharides and these all culture kept in growth room.

6.8 : Results and Discussion:

Rice flour:

Table 6.1: Optimized media gelling obtained on combination of Agar 3gm+ Rice flour 50gm (Plate 6.7, A-D)

Sr No.	Agar (gm) + Rice flour (gm)	Result
1.	1.0 + 50.0	Slightly gel
2.	2.0 + 50.0	Slightly gel
3.	**3.0 + 50.0**	**Gel**
4.	4.0 + 50.0	Gel
5.	5.0 + 50.0	Gel
6.	6.0 + 50.0	Gel
7.	7.0 + 50.0	Gel

Sr No.	Agar (gm) + Rice flour (gm)	Result
1.	3.0 + 10.0	Not gel
2.	3.0 + 20.0	Not gel
3.	3.0 + 30.0	Not gel
4.	3.0 + 40.0	Slightly gel
5.	**3.0 + 50.0**	**Gel**

Table 6.2: Optimized media gelling obtained on combination of Agar 4gm+ Isubgol 25gm (Plate 6.6, A-D).

Isubgol:

Sr No.	Agar (gm) + Isubgol (gm)	Result
1.	1.0 + 25.0	Not gel
2.	2.0 + 25.0	Slightly gel
3.	3.0 + 25.0	Slightly gel
4.	**4.0 + 25.0**	**Gel**
5.	5.0 + 25.0	Gel
6.	6.0 + 25.0	Gel
7.	7.0 + 25.0	Gel

Sr. No.	Agar (gm) + isubgol (gm)	Result
1.	4.0 + 20.0	Slightly gel
2.	**4.0 + 25.0**	**Gel**
3.	4.0 + 30.0	Gel

Table 6.3: Optimized media gelling obtained on combination of Agar 4gm+ Natural gum 10gm (Plate 6.4, A-D)

Natural gum:

Sr No.	Agar + natural gum(gm)	Result
1.	1.0 + 10.0	Not gel
2.	2.0 + 10.0	Slightly gel
3.	3.0 + 10.0	Slightly gel
4.	**4.0 + 10.0**	**Gel**
5.	5.0 + 10.0	Gel
6.	6.0 + 10.0	Gel
7.	7.0 + 10.0	Gel

Sr No.	Agar + natural gum (gm) (gm)	Result
1.	**4.0 + 10.0**	**Gel**
2.	4.o + 20.0	Gel
3.	4.0 + 30.0	Gel

Table 6.4: Optimized media gelling obtained on combination of Agar 4gm+ Sabudana 10gm (Plate 6.5, A-D)

Sabudana:

Sr No.	Agar + Sabudana (gm) (gm)	Result
1.	1.0 + 10.0	Not gel
2.	2.0 + 10.0	Slightly gel
3.	3.0 + 10.0	Slightly gel
4.	**4.0 + 10.0**	**Gel**
5.	5.0 + 10.0	Gel
6.	6.0 + 10.0	Gel
7.	7.0 + 10.0	Gel

Sr No.	Agar + sabudana (gm) (gm)	Result
1.	**4.0 + 10.0**	**Gel**
2.	4.0 + 20.0	Gel
3.	4.0 + 30.0	Gel

Table 6.5: Optimized media gelling obtained on combination of Agar 4gm+ Suji 10gm. (Plate 6.3, A-D)

Suji:

Sr No.	Agar (gm) + Suji (gm)	Result
1.	1.0 + 10.0	Not gel
2.	2.0 + 10.0	Slightly gel
3.	3.0 + 10.0	Slightly gel
4.	**4.0 + 10.0**	**Gel**
5.	5.0 + 10.0	Gel
6.	6.0 + 10.0	Gel
7.	7.0 + 10.0	Gel

Sr No.	Agar (gm) + suji (gm)	Result
1.	**4.0 + 10.0**	**Gel**
2.	4.0 + 20.0	Gel
3.	4.0 + 30.0	Gel

Table 6.6: Optimized media gelling obtained on combination of Agar 3gm+ Guar gum 25gm.(Plate 6.1, A-D)

Guar gum:

Sr N0.	Agar (gm) + guar gum (gm)	Result
1.	1.0 + 25.0	Not gel
2.	2.0 + 25.0	Slightly gel
3.	**3.0 + 25.0**	**Gel**
4.	4.0 + 25.0	Gel
5.	5.0 + 25.0	Gel
6.	6.0 + 25.0	Gel
7.	7.0 + 25.0	Gel

Sr No.	Agar (gm) + guar gum (gm)	Result
1.	3.0 + 20.0	Slightly gel
2.	**3.0 + 25.0**	**Gel**
3.	3.0 + 30.0	Gel

Table 6.7: Optimized media gelling obtained on combination of Agar 4gm+ Singoda flour 30gm (Plate 6.2, A-D)
Singoda flour:

Sr No.	Agar + singoda flour(gm)	Result
1.	1.0 + 30.0	Not gel
2.	2.0 + 30.0	Slightly gel
3.	3.0 + 30.0	Slightly gel
4.	**4.0 + 30.0**	**Gel**
5.	5.0 + 30.0	Gel
6.	6.0 + 30.0	Gel
7.	7.0 + 30.0	Gel

Sr No.	Agar (gm) + singoda flour (gm)	Result
1.	4.0 + 10.0	Slightly Gel
2.	4.0 + 20.0	Slightly Gel
3.	**4.0 + 30.0**	**Gel**

Table 6.8: Summary of above experiments (best gelling combination) and its costing.

Sr No.	Trial	Combination	Result	Rates/kg	Rs/gm	Media cost/liter	Cost/liter	Cost/bottle
1.	A+RF	3gm + 50gm	GEL	24.00/Kg	2paisa/gm	4.8 + 1.0	5.80	0.29 paisa
2.	A+IS	4gm + 25gm	GEL	588.00/Kg	60paisa/gm	6.4 + 15.0	21.4	1.07
3.	**A+NG**	**4gm + 10gm**	**GEL**	**100.00/Kg**	**20paisa/gm**	**6.4 + 2.0**	**8.4**	**0.42 paisa**
4.	A+SD	4gm + 10gm	GEL	150.00/Kg	15paisa/gm	6.4 + 1.5	7.9	0.39 paisa
5.	A+SJ	4gm + 10gm	GEL	34.00/Kg	3paisa/gm	6.4 + 0.30	6.70	0.33 paisa
6.	A+GG	2gm + 10gm	GEL	300.00/Kg	30paisa/gm	3.2 + 9.0	12.2	0.61 paisa
7.	A+SN	4gm + 30gm	GEL	160.00/Kg	16paisa/gm	6.4 + 4.8	11.2	0.56 paisa

Table 6.9: Observations

Sr. No.	Date of Experiment	Combination	TotalNo. of Ex-plant (banana)	Observation(motility)		
				7th Day	14th Day	21st Day
1.	21.06.2012	Agar+ Rice flour	25	00	00	00
2.	21.06.2012	Agar+ Isubgol	15	15	15	15
3.	21.06.2012	Agar+ Natural Gum	05	05	05	05
4.	21.06.2012	Agar+Sabudana	25	25	25	25
5.	21.06.2012	Agar+ Suji	20	10	10	10
6.	25.06.2012	Agar+ Guargum	20	10	00	00

7.	25.06.2012	Agar+ Natural Gum	25	25	25	25
8.	25.06.2012	Agar+ Rice flour	05	05	05	05

Conclusion:

Instead of only agar if we use natural gum along with agar it will cost only Rs. 1260 for one day for 150 l media as compared to agar if used individually, it will cost Rs. 1680.

Time Period	**Amount of Media (liter)**	**Amount of Agar Required (in kg)**	**Agar cost (Rs./)**	**Cost of Agar + Natural Gum (Rs./)**
1 Day	150 liter	1.05 Kg	1680	1260
30 Days	4500 liter	31.5 Kg	52,920	37,800
180 Days	27,000 liter	189 Kg	3,02,400	2,26,800

Plate : 6.1

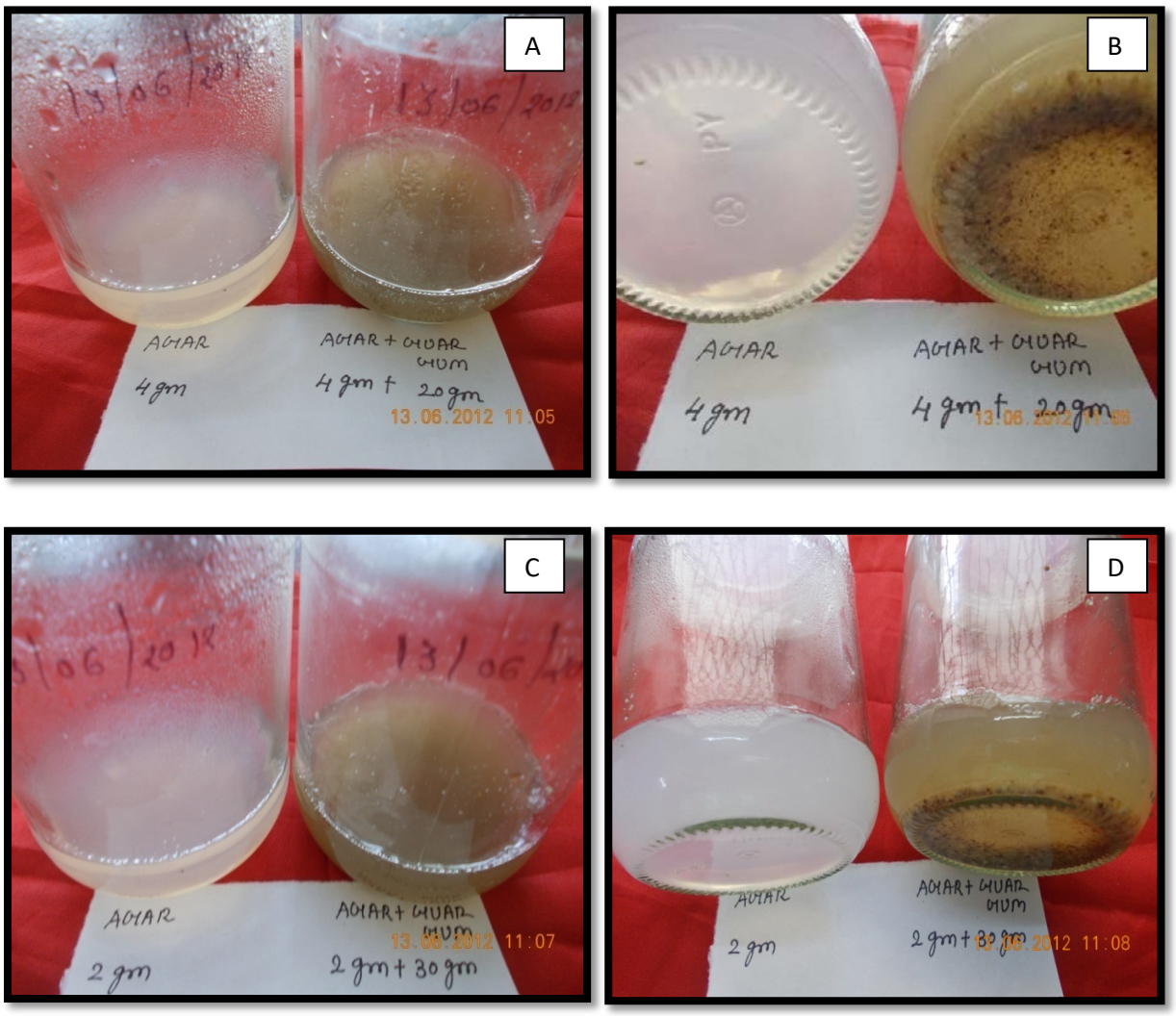

Plate 6.1: A, B, C, D showing gelling stage in combination of Agar-Agar+Guar Gum (*Cyamopsis tetragonoloba*)

Plate : 6.2

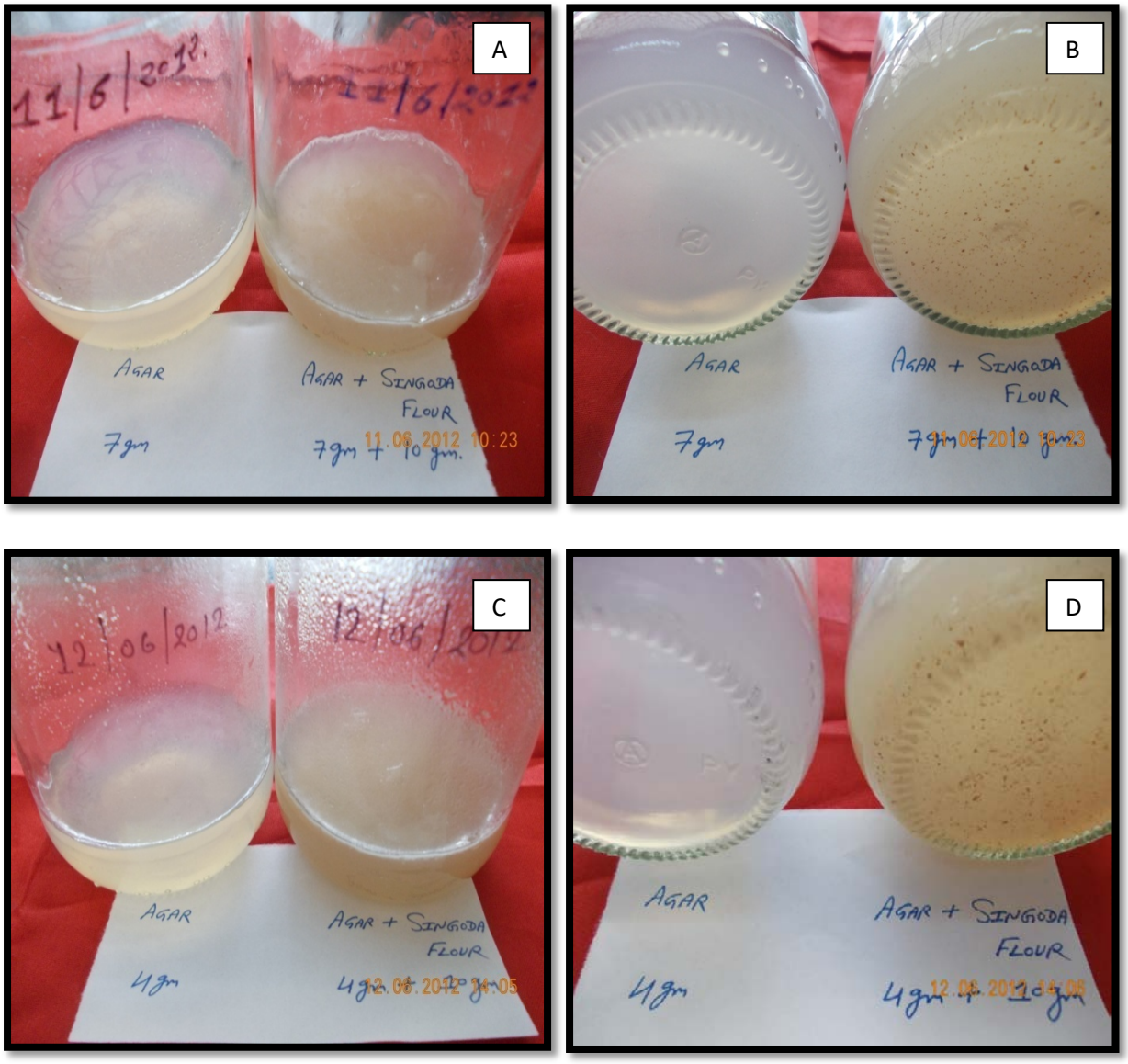

Plate 6.2: A, B, C, D showing gelling stage in combination of Agar-Agar+ Singoda flour (*Trapa bispinosa*)

Plate: 6.3

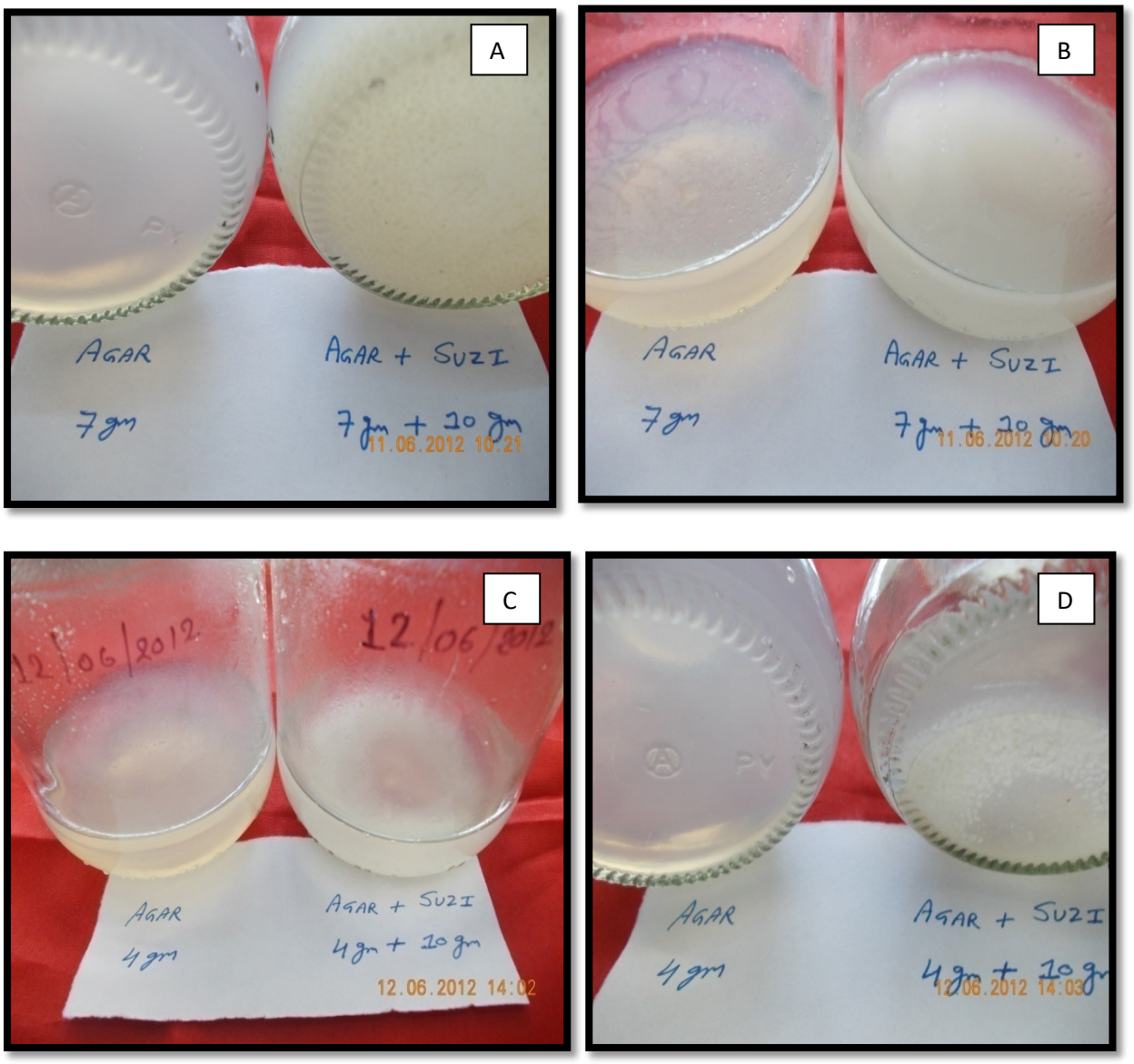

Plate6.3: A, B, C, D showing gelling stage in combination of Agar-Agar + Suji

Plate : 6.4

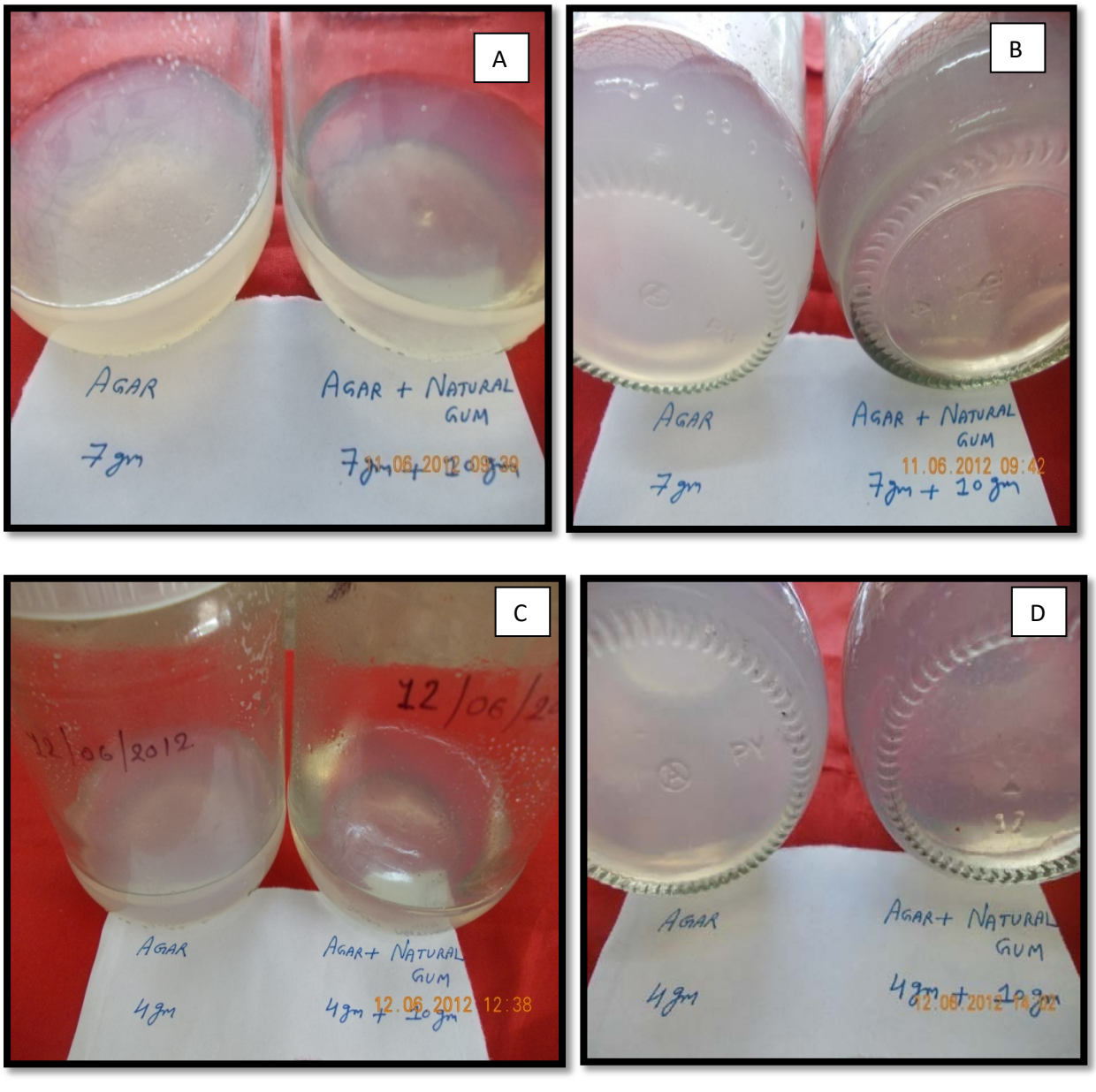

Plate6.4: A, B, C, D showing gelling stage in combination of Agar-Agar+ Natural Gum

Plate :6.5

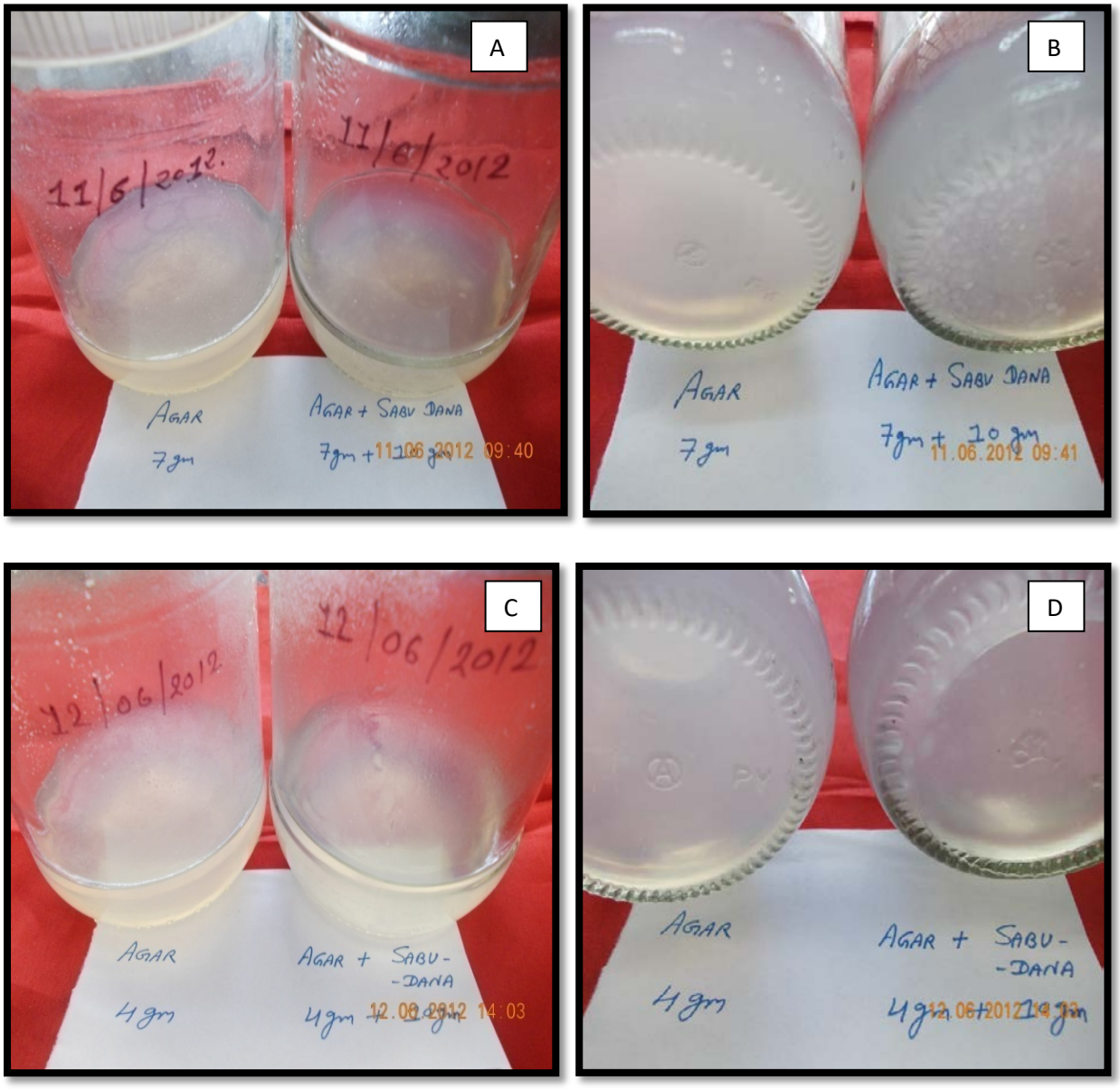

Plate6.5: A, B, C, D showing gelling stage in combination of Agar-Agar+ Sabudana

Plate : 6.6

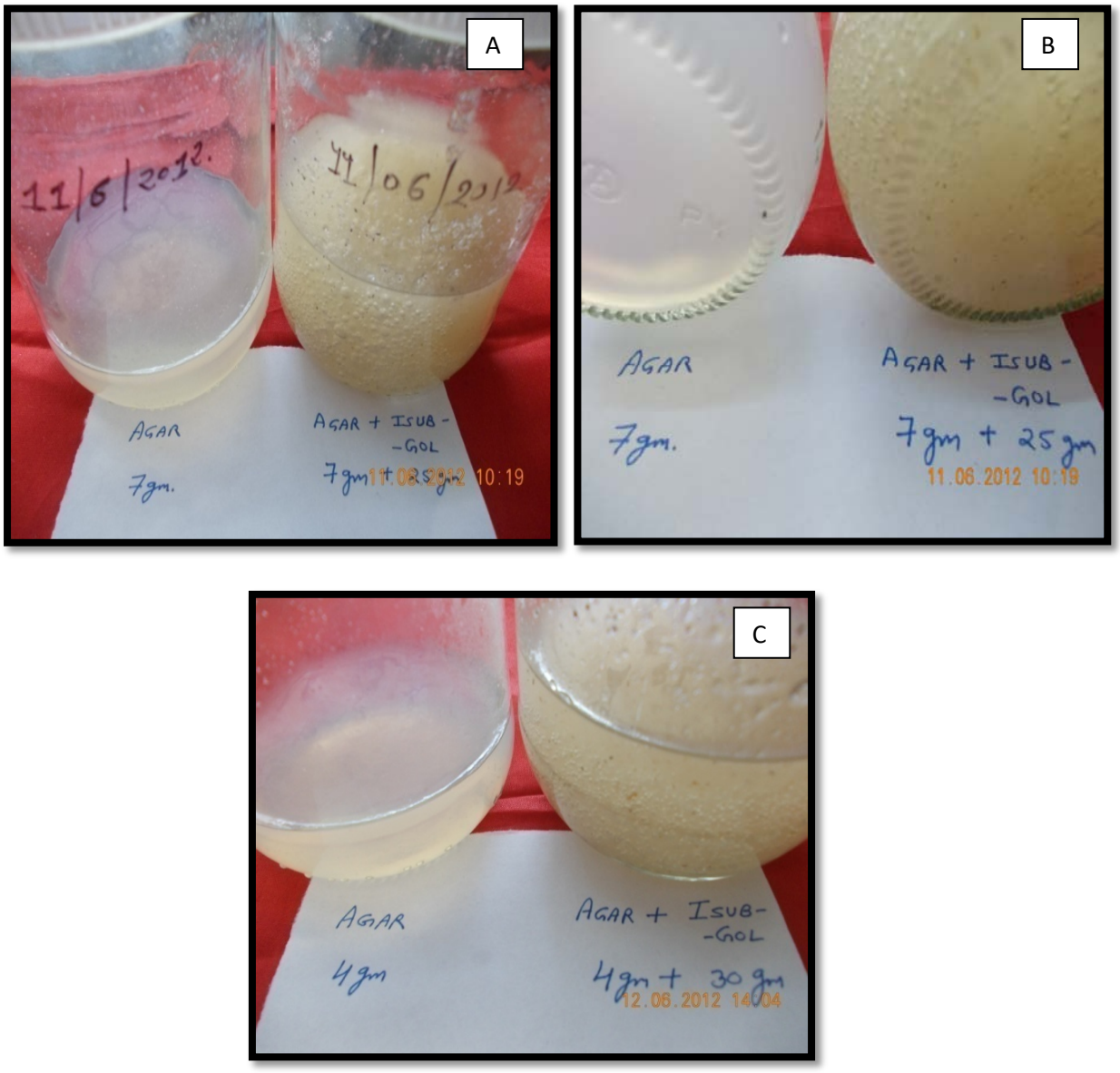

Plate 6.6: A, B and C showing gelling stage in combination of Agar-Agar+ Isubgol

Plate : 6.7

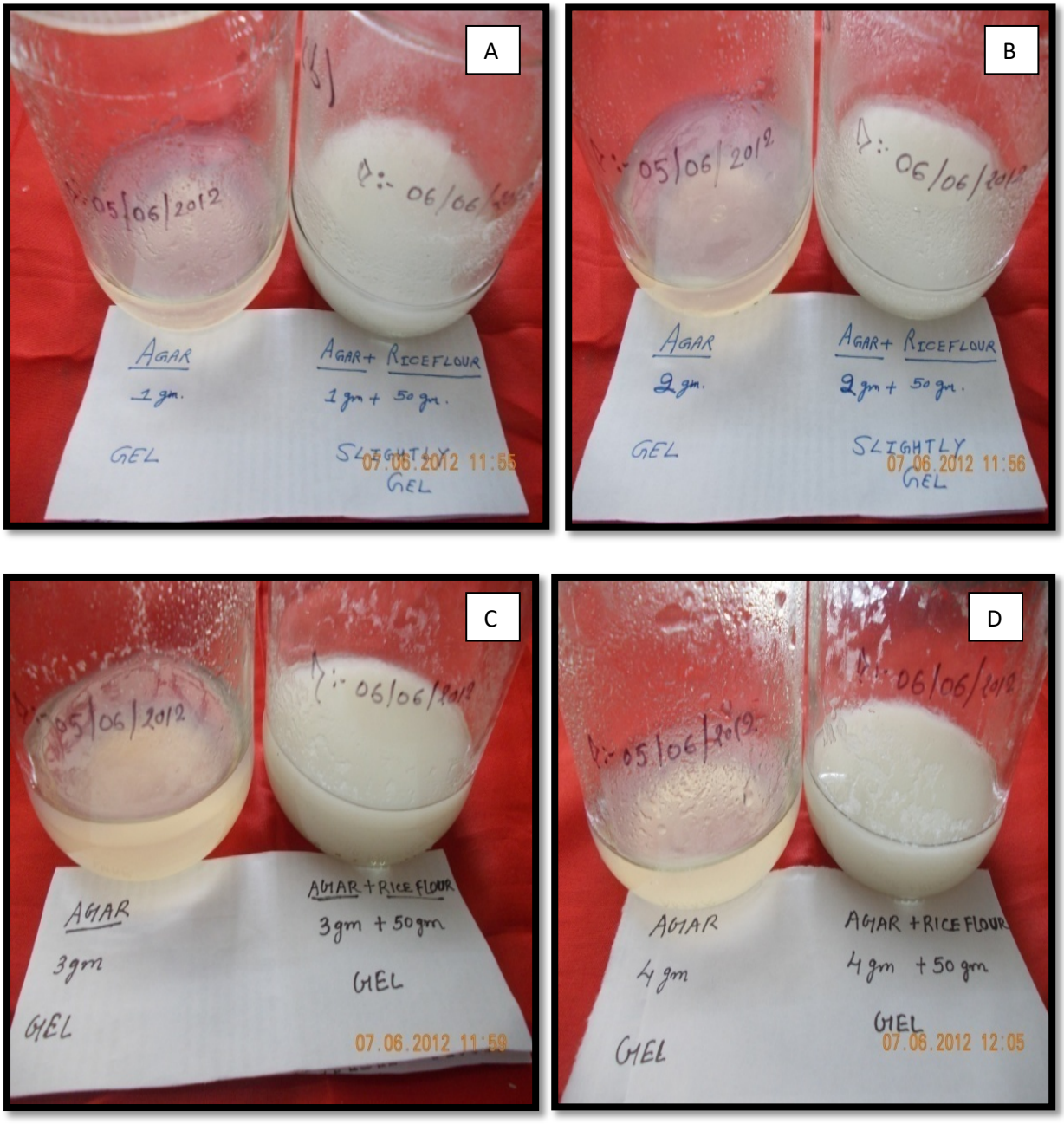

Plate: 6.7:A, B, C, D showing gelling stage in combination of Agar-Agar+ Rice flour