Extraction of agar from locally grown *Gracilaria verrucosa* and development of gelatin free set-yoghurt product using agar

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Abstract

Seaweed agar is an important healthy food item, though it is not extracted at commercial level in Sri Lanka. This study investigated an agar extraction method that render high agar yield and development of agar incorporated gelatin free yoghurt targeting the needs of vegetarian communities. The technologies developed, in this study, to extract food grade agar from locally available *Gracilaria verrucosa* and to produce agar incorporated yoghurt which has potential to commercialize as an industry. Agar was extracted using *G. verrucosa* under optimum conditions: Dried *G. verrucosa* was soaked in an acetic acid solution at pH 5 for 30 min; soaked *G. verrucosa* was pressure-cooked with 45 times volume of water for 20 min; the agar extract was allowed to set in trays for 6 h at 25±2 °C; the resulted gel layers were frozen for 8 h and thawed for 4 h at 25±2 °C. The melted water was drained out and gel layers were cut into strips. Gel strips were dried at 45 °C for 36 h and dried agar were ground to obtain a fine agar powder. A gelatin free set-yoghurt product was developed using extracted agar as a texture stabilizer. The developed yoghurt (0.25% agar) which scored high for sensory quality attributes, showed similar sensory properties as in gelatin (0.61%) containing yoghurt ($p>0.5$). The pH and titratable acidity of the seaweed yoghurt were 4.5 and 0.85% (w/w) respectively on 15th day of storage at 4±2 °C. Agar extracted from *G. verrucosa* contained 80.1% (w/w) of dietary fiber. It was found that seaweed yoghurt contains 0.18% (w/w) of dietary fiber content. Agar contained set yoghurt consists of 77.34, 3.40, 3.10, 0.75 and 22.66% (w/w) of moisture, protein, fat, total ash and total solid content, respectively.

Keywords: *G. verrucosa*, Agar, Gelatin free, Set-yoghurt

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Introduction

Seaweeds are macroscopic algae, which form a significant component of the marine living resources. Based on the colour pigmentation seaweeds are broadly categorized into green (Chlorophyta), brown (Phaeophyta), red (Rodophyta) and blue green algae (Mohamed *et al.,* 2012). Seaweeds play an important role in producing polysaccharides such as agar, carrageenan and alginic acid which are considered as important food additives.

Gracilaria sp., Gelidium sp., Saragassum sp., Turbinaria sp., Ulva sp. and *Caulerpa sp.* have been identified as economically valuable seaweed species in Sri Lanka. The seaweed production in Sri Lanka mainly comes from the wild collection. Recently some pilot scale projects were initiated for culture of seaweeds with community participation but its contribution to the total production is negligible yet. The current *G. verucosa* wild collection predominantly comes from Kinniya in Trincomalee. Naturally grown *G. verucosa* can be seen at near shore in Trincomalee, Sri Lanka.

Agar is considered as a gel-forming substance obtainable from certain species of red seaweeds called "agarophytes" composed of neutral gelling molecules, agarose and to lesser extent acidic non-gelling molecules, agaropectin. *Gracilaria* is the most abundant and promising resource for agar production (McHugh, 1987). Agar has a wide variety of uses in the industries such as bakery, confectionary, dairy, pharmaceutical, biomedical and other fields (McHugh, 1987). The World Health Organization (WHO) permits agar for use in the human food industry and it has also been accepted and authorized by the regulations of various countries including the United Kingdom, Federal Republic of Germany, Union of Socialist Republics, France and Poland (McHugh, 1987). The Food and Drug Administration (FDA) of the United States assigns the agar a grading of Generally Recognized As Safe (GRAS). (McHugh,1987).

Presently, gelatin free set-yoghurt products that come under ready-to-eat cultured dairy products are not commercially manufactured in Sri Lanka and also not available in local markets. Dynamic rheological experiments showed that yoghurts with added gelatin exhibits more solid like behavior than the yoghurts prepared without it (Fiszman, *et al.*, 1999). Therefore almost all of the Sri Lankan yoghurt manufacturers use gelatin as a

texture stabilizer. As gelatin is animal origin, knowledgeable vegetarian community rejects this nutritious cultured dairy product from their consumption.

The aim of this study was to develop a method to extract agar from *G. verrucosa* and to develop a ready to eat, seaweed agar incorporated set-yoghurt product that has similar textural properties to gelatin containing set-yoghurt, targeting the vegetarian community.

Materials and Methods

Collection and Preparation of *G. verrucosa* prior to extraction

G. verrucosa plants were collected from Kinniya, Trincomalee, Sri Lanka using a scoop net. Collected plants were sorted and debris and other extraneous matter were removed from the moss and washed using seawater. Sorted *G. verrucosa* plants were sun dried for 2-3 days in order to deteriorate colour pigments. Dried plants were washed and sundried repeatedly for 3 times until a light yellow coloured clean plants were obtained.

Development of agar extraction method

Based on Food and Agriculture Organization (FAO), (1987) agar was extracted by a modified procedure in order to develop a convenient and suitable technique to establish agar extraction as an industry in Sri Lanka.

Chemical pre-treatment

Dried plants of *G. verrucosa* were treated with the solutions with different pH values. Acidic pH values were obtained by using artificial vinegar (Acetic acid) and alkali pH values were obtained by using NaOH (AR Grade/ Sigma-Aldrich). Seaweeds were soaked in the solutions with different pH values for 30 min. The seaweeds were then washed two or three times to free it from acid or alkali and transferred to the cooking vessel for extraction. The soaking pH that resulted in the maximum agar yield with better quality was selected as the optimum soaking pH.

Extraction

Agar extraction was performed using different seaweed: water ratios (1: 40; 1:45; 1:50) and the yield and the ash content of dried agar powder were selected as the efficiency analyzing criteria. Pressure cooking and open vessel cooking were tested with different

time combinations to find the optimum cooking method and optimum time that yields maximum amount of agar.

Filtration and the gelation

On completion of the extraction, the hot aqueous extract was filtered using a cheese cloth and a screw press. The hot aqueous agar extract was transferred to stainless steel or plastic trays making a gel layer of 2 cm thickness.

Separation of agar from hot solution

To remove the water from the extract freeze, thaw method was used. The optimum freezing time was selected based on the ash (impurities) percentage of dry agar powder. Water soluble impurities were get removed with water that is drained off when thawing.

Drying

On completion of draining the water, the agar layers were cut in to strips and dried in a drying cabinet. The optimum temperature and time combination that results moisture content of <15% was found.

Milling

The dried strips were milled using a home scale grinder until obtain a powder of ≤200 µm (Fritsch Stainless Steel Sieve).

Characterization of agar powder

The colour of the powder was decided using the Munsell Colour Guide 2005 (Munsell Colour Science Laboratory, Rochester Institute of Technology). Moisture, dry matter and ash content and were analyzed as described in the AOAC, 1995. Total fibre content was analyzed using enzymatic gravimetric method (Asp *et al.*, 1983). Gel strength of 1.5% gel was measured using Instron Texture Analyzer 4465. Melting point of 1.5% gel was measured using method as described by Marshall *et al.*, 1949. The sol-gel transition temperature of 1.5% agar solution was measured as described by Esquivel *et al.*, 2008.

Development of agar incorporated set yoghurt

Development of yoghurt was carried out in Research and Development Laboratory of Milco (Pvt) Ltd, Narahenpita, Sri Lanka. Standardized cow's milk (2.5% fat, 8.3% solid

nonfat) was obtained from the yoghurt plant of Milco (Pvt) Ltd. Agar powder extracted from *G. verrucosa,* sugar, Highland (Brand) full cream milk powder, egg yolk colour (E102, E122), vanilla essence and the freeze dried yoghurt culture (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*) produced by Veterinary Research Institute, Peradeniya, Sri Lanka were used as the other ingredients for yoghurt production. Trial and error method was used to formulate the set yoghurt product that has similar textural properties as gelatin containing set yoghurt. The process of yoghurt production was altered in order to obtain agar incorporated set yoghurt with favorable sensory properties (appearance, aroma, texture and flavour). Both product and market oriented sensory evaluations were conducted using 30 in-house panelists. Gelatin containing yoghurt was prepared according to the recipe used in Milco (Pvt) Ltd. as the reference for market oriented sensory evaluation.

Table 1. Formulations used for the sensory evaluation

Ingredient (%)	T1	T2	T3	T4	T5	T6	Gelatin yoghurt
Standardized cow's milk	84.7	85.0	85.1	85.03	85.05	85.06	84.7
Full cream milk powder	3.6	3.6	3.6	3.6	3.6	3.6	3.6
Sugar	11.0	11.0	11.0	11.0	11.01	11.01	11.0
Agar extract (*G. verrucosa*)	0.61	0.31	0.21	0.27	0.25	0.23	-
Gelatin	-	-	-	-	-	-	0.61
Vanilla essence	0.025	0.025	0.025	0.03	0.03	0.03	0.025
Egg yolk colour	0.065	0.065	0.065	0.07	0.07	0.07	0.065

T- Treatment

Chemical analysis

The moisture, dry matter, ash, crude protein and crude fat were analyzed by proximate composition analysis as described in the AOAC, 1995.

Determination of physical properties

pH of the yoghurt was tested using a pH meter (HANNA-pH 210, Germany), Titratable acidity (%) (w/v) was measured by titrating with a 0.1 N NaOH solution. The Syneresis Index was measured as described in Farooq and Haque, 1992.

Evaluation of shelf life

The sensory evaluation (organoleptic properties), pH value, titratable acidity, syneresis, microbiological examinations (presence and counts of Coliform and *E.coli,* yeast and mould counts) were carried out for the shelf life study. Both gelatin containing yoghurt and developed seaweed agar incorporated yoghurt samples were prepared manually at Research and Development Laboratory under the same conditions and same day for the shelf life study. All the analysis was carried out on 1^{st}, 5^{th}, 10^{th}, 15^{th} and 20^{th} days from production.

Coliform/ *E.coli* count for yoghurt

Petri films (3M) were used to get the *E.coli* and Coliform counts. Colonies were counted using the colony counter (Gallenkamp CNW-300-030G Semi-Automatic).

Yeast and mould counts

Enumeration of yeast and mould counts was carried out according to the SLS 516 Part 2: 1991. One milliliter from 10^{-1}dilution of the sample was placed in the sterile petri dishes under aseptic conditions. About 15 mL of potato dextrose agar at 45±1 ºC was poured into each petri dish followed by rotating the closed petri dishes (pour plate method).The agar was allowed to solidify at room temperature inside the laminar floor under aseptic conditions. Petri dishes were incubated (Gallenkamp, model INA-300, UK) aerobically in an inverted position at 25±1 ºC for 5 days. The colonies were counted using a colony counter in subdued light and the results were expressed as "yeast and mould colony forming units" (CFU) per 1 gram of the sample.

Statistical Analysis

Statistical analysis was carried out using Friedman test of Minitab 16 Software. Significant differences were determined at $p<0.05$ level.

Results

Effect of chemical pre-treatment on the agar yield

According to Coppen and Nambiar (1991) chemical treatment of agarophytes prior to extraction often produces a better agar in terms of quality or yield than the agar produced without such treatments. Quality of agar is measured by means of gel strength, setting temperature and melting temperature.

Table 2. Agar yield and quality under different soaking pH values (pretreatment) while other processing conditions at constant

pH	Average yield of dry agar powder%	Setting temperature of 1.5% agar solution (°C)	Melting point of 1.5% agar gel (°C)	Gel strength of 1.5% agar gel (g/cm^2)
3.0	34.15±0.5	39.0±0.42	86.5±0.21	775.88±4.43
5.0	37.25 ±0.8	38.6±0.56	86.3±0.82	793.67±10.0
6.0	22.40±1.0	39.0±0.41	85.2±0.92	780.83±2.62
7.0	20.15±0.5	40.5±0.32	86.4±0.27	782.42±3.47
12.5	13.95±0.2	39.9±0.52	86.8±0.61	805.31±3.67
12.6	12.65±0.2	37.8±0.48	87.5±0.31	804.21±3.67
12.7	10.70±0.3	37.2±0.36	87.3±0.81	805.58±3.67

Constant conditions: Ratio of dried seaweed to water (1:40), pressure cooking of seaweed for 20 min under low flame, freezing of gel layers for 8 h (-18±2°C), thawing of frozen gel layers for 4 h at room temperature, drying of thawed gels at 50±5°C for 36 h in a drying cabinet and pH 5.0 was selected as the optimum pH of chemical pre treatment

Table 3. Changes in yield and ash content of agar powder with different ratios of dry seaweed to water while other processing conditions at constant

Ratio of dry seaweed to water (g)	yield of dry powder (%)	Ash content of dry powder (%)	Nature of gel after setting for 6 h
1: 40	37.25±0.3	2.11±0.03	2 cm thick gel layers can be removed from the tray as a whole layer without breaking the gel.
1: 45	38.78±0.8	1.96±0.02	-do-
1: 50	39.24±0.4	1.88±0.03	2 cm thick gel layers are not properly set and cannot be removed from the tray as a whole layer.

Constant conditions: Soaking of seaweed at pH 5, pressure cooking of seaweed for 20 min under low flame, freezing of gel layers for 8 h (-18±2°C), thawing of frozen gel layers for 4 h at room temperature, drying of thawed gels at 50±5°C for 36 h in a drying cabinet and Ratio of seaweed to water at 1:45 g was selected as the best dilution factor.

Table 4. Effect of cooking method and time while other processing conditions at constant

Cooking method	Time (min)	Yield of dry powder %
Cooking in an open pan with water	20	31.33±0.68
Pressure cooking	10	34.67±0.78
Pressure cooking under low flame	20	38.45±0.80

Constant conditions: Soaking of seaweed at pH 5, Ratio of dried seaweed to water (1:40), freezing of gel layers for 8 h (-18±2°C), thawing of frozen gel layers for 4 h at room temperature, drying of thawed gels at 50±5°C for 36 h in a drying cabinet and pressure cooking under low flame for 20 min was selected as the optimum cooking condition

Table 5. Effect of different freezing times on ash content of dry agar powder, while other processing conditions are at constant

Freezing time at -18 °C (h)	Ash percentage of dry agar-agar powder
2	11.03±0.25
4	9.08±0.32
6	3.55±0.61
8	2.86±0.72
10	2.75±0.52
12	2.53±0.37
17	2.23±0.89

Constant conditions: Soaking of seaweed at pH 5, Ratio of dried seaweed to water (1:40), pressure cooking of seaweed for 20 min under low flame, thawing of frozen gel layers for 4 h at room temperature, drying of thawed gels at 50±5°C for 36 h in a drying cabinet and eight hour freezing time was selected as the cost effective optimum freezing time.

Table 6. Specifications of *G. verrucosa* agar-agar powder extracted from the developed method

Parameter	Value	Recommended range
Colour	8/2.5 Y	-
Moisture content	17.45±0.1% (w/w)	Max 18%
Total ash	2.07±0.1% (w/w)	Max 6.5%
Total fibre	80.10±0.7% (w/w)	-
Gel strength (1.5% gel at 25°C)	793.67±10 g/cm^2	700-1000 g/cm^2
Melting point of 1.5% gel	86.3±0.82 °C	85-95 °C
Setting temperature	38.6±0.56 °C	32-45 °C
Solubility	2 .5 min under medium power of a microwave oven (1.5% agar solution)	Soluble in boiling water

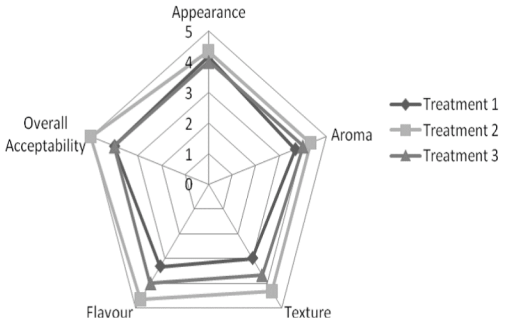


Fig. 1. Spider web diagram of step 1 of agar incorporated yoghurt development

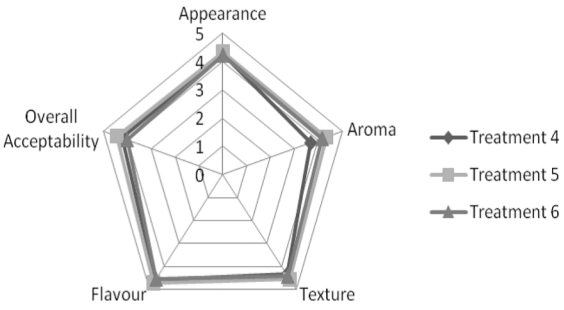


Fig. 2. Spider web diagram of step 2 of agar incorporated yoghurt development

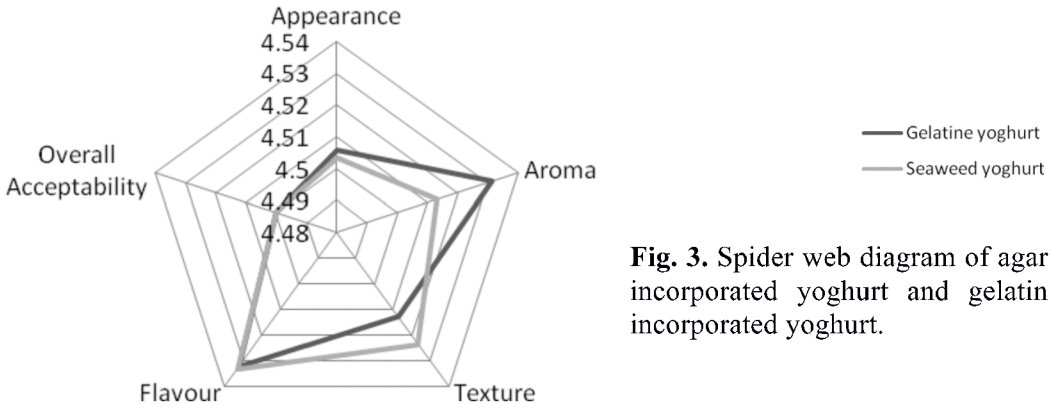


Fig. 3. Spider web diagram of agar incorporated yoghurt and gelatin incorporated yoghurt.

Shelf life determination of agar incorporated yoghurt

Table. 7. Microbial test results of seaweed agar incorporated yoghurt and gelatine yoghurt during the storage trial (4±2°C)

Parameter (CFU/g)	Seaweed agar incorporated yoghurt				Gelatin yoghurt			
	Day 1	Day 5	Day10	Day15	Day1	Day 5	Day 10	Day15
Coliform count	0	0	0	0	0	0	0	0
E.coli count	0	0	0	0	0	0	0	0
Yeast	10	50	80	80	20	30	30	50
Mould count	0	0	0	0	0	0	0	0

Microbiological counts of both seaweed agar incorporated yoghurt and gelatin yoghurt were complied with SLSI standards (P<0.5) during 15 days of storage time.

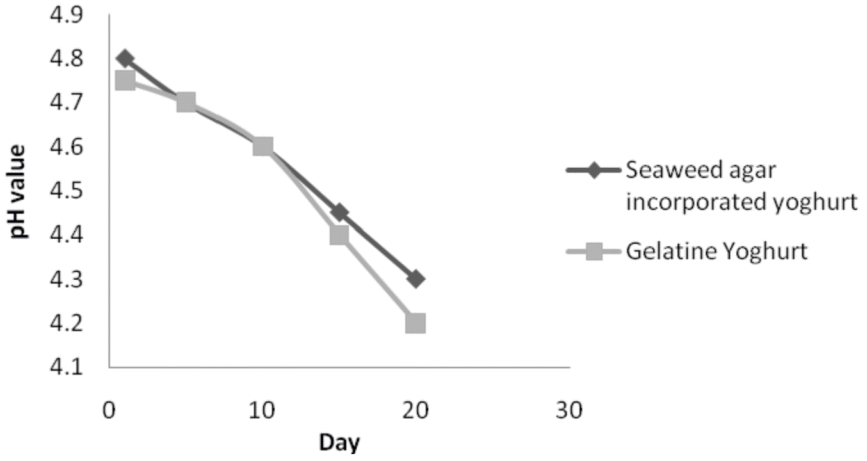


Fig. 4. Variation of the pH value of the seaweed agar incorporated yoghurt and gelatin yoghurt over the experimental period.

The variation in pH of both seaweed agar incorporated yoghurt and gelatin yoghurt is illustrated in Fig. 4 which was measured at 5 days interval over 20 days.

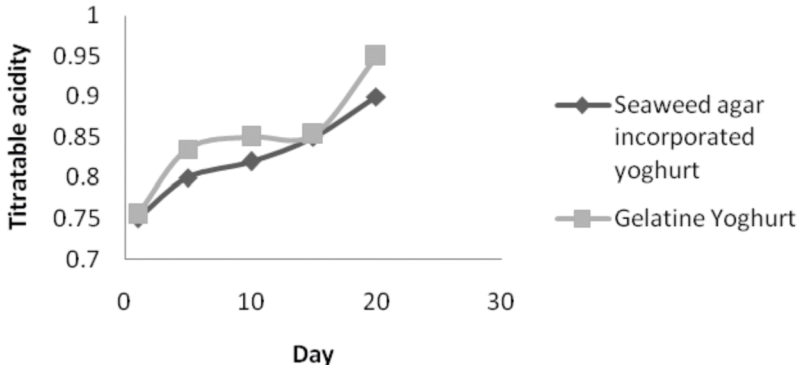


Fig. 5. Variation in the titratable acidity of the yoghurt over the experimental period

The variation in the titratable acidity of both yoghurt types produced on same day under same conditions and stored in the same refrigerated conditions were measured at 5 days interval over 20 days has been illustrated in the Fig. 5.

Table. 8. A comparison of syneresis of agar incorporated yoghurt and gelatin containing set yoghurt on 15th day of storage

Type of yoghurt	Percentage of Syneresis (w/w)
Gelatin yoghurt	23.32
Seaweed agar incorporated yoghurt	20.15

Table. 9. Proximate composition of developed seaweed agar incorporated yoghurt

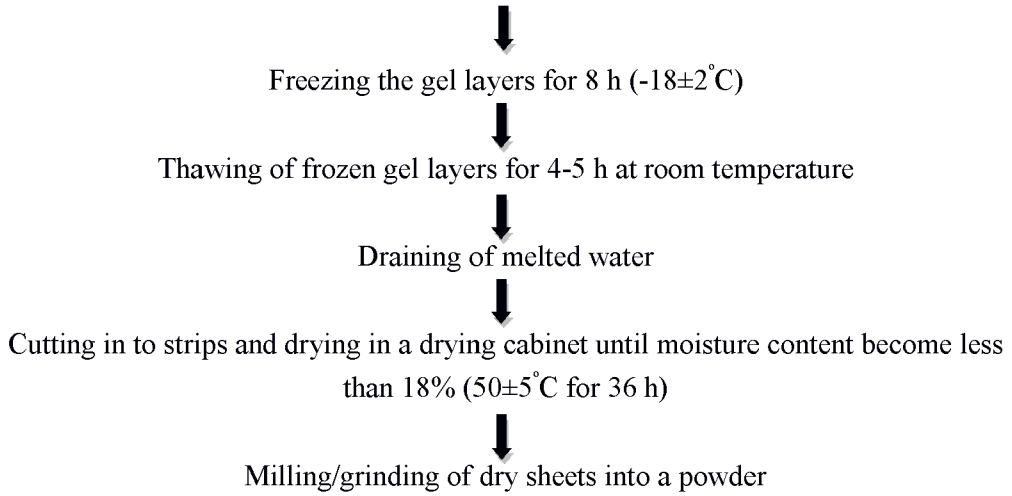
Component	Available %
Fat	3.10
Total solids	22.66
Moisture	77.34
Protein	3.4
Ash	0.75
Dietary fibre	0.18

The highest agar yield was obtained from the dried moss of *G. verrucosa* pre-treated with acidic solution of pH 5. Seaweed agar extracted after alkaline treatment has shown higher gel strength values than the vinegar treated seaweed gels. Melting point and

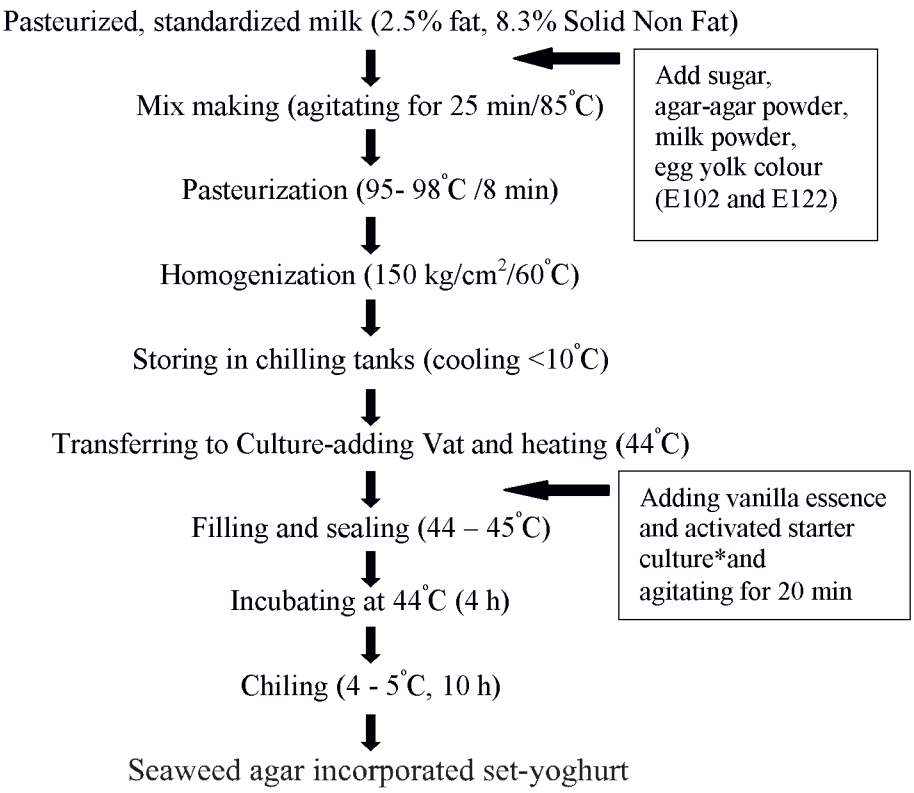
setting temperature of 1.5% agar gels extracted after different chemical treatments were not significantly different ($p>0.5$). The building material of agar is polysaccharide and ash content of dried agar powder indicates the content of impurities. Water soluble impurities can be reduced by maximizing the water amount used for cooking. When thawing of frozen gel layers all the water soluble impurities are drained off. Use of maximum amount of water enhances the agar yield. However if the water content exceeds the limit the gel, layers cannot be handled properly as a whole layer. Agar is trapped in the cell walls of *G. verrucosa* and pressure cooking may support in rupturing of cell walls and expedite more agars in to the solution than cooking in an open pan. Freeze-thaw method used to purify the agar by removing water soluble impurities and it helps in the drying process by reducing the drying time (less time is needed to dry when the moisture content of gel is less). As freezing is costly minimum time that results less ash content was selected as the optimum freezing time. Ash content of dried agar powder produced using the freezing times of 8, 10, 12, 17 h are not significantly different ($p>0.5$). Therefore 8 h was selected as the cost effective optimum freezing time.

Developed agar-agar extraction process

G. verrucosa
(Collected from natural seaweed beds at Trincomalee)
⬇
Washing and sun bleaching
(Repeated 3 times until obtain light yellow colour pure material)
⬇
Soaking in a pH 5 acetic acid solution for 30 min
⬇
Neutralization by washing with running water
⬇
Addition of water to soaked seaweeds
(45 times based on initial dry weight of seaweeds)
⬇
Pressure cooking of seaweeds for 20 min under low flame
⬇
Filtering the cooked seaweed mass through a cheese cloth using a screw press
⬇
Allowing setting seaweed sols for 6 h to retain layers (2 cm thickness) in aluminum trays under room temperature



Production process flow of seaweed yoghurt



Sensory Evaluation

According to the spider web diagram (Fig.1) Treatment 2 has scored the maximum score for sensory attributes and it was selected from Step 1. In Step 2 three different percentages of seaweed agar between T2 and T3 was taken.T5 was selected as the best formula (Fig.2).

According to the spider web diagram (Fig.4) and statistical analysis flavour, appearance and overall acceptability of both agar yoghurt and gelatin yoghurt were not significantly different ($p>0.5$). Texture of the agar yoghurt was better than the gelatin yoghurt and bit more preference was gained by the aroma of gelatin yoghurt than the aroma of agar yoghurt. As overall acceptability level was more or less similar. T5 was selected as the final formula of agar incorporated yoghurt.

Microbiological results

According to the SLS 516: Part 2, when consider about the hygienic quality it should be free from coliforms, less than 1000/g yeasts and less than 1/g moulds (SLS, 1991). The microbiological counts of seaweed agar incorporated yoghurt (Table 7) were complied with the SLSI standards during 16 days. On the 15th day of storage only 80 CFU was obtained as the yeast count.

Variation of pH of yoghurt during the refrigerated storage

pH value was declined throughout the storage period of the both seaweed yoghurt and gelatin yoghurt (Fig.4). On 20th day sour taste and unpleasant acidic odor was observed and therefore both yoghurt types were not suitable for consumption. pH declining pattern of both seaweed agar incorporated yoghurt and gelatin yoghurt was not significantly different ($P<0.5$) and hence shelf life of the seaweed agar incorporated yoghurt was taken as 16 days.

Variation of titratable acidity and pH of yoghurt during the refrigerated storage

Titratable acidity of both yoghurts increased throughout the storage period (Fig.5). But acidity values (0.75-0.9) were within the recommended range for titratable acidity of yoghurt which is 0.8 – 1.25% according to the SLS 735: Part 2 (1987). Therefore the shelf life of the yoghurt was confirmed as 16 days as pH value was also in the recommended range.

Syneresis

The syneresis of gelatin yoghurt (23.32%) was higher than the agar incorporated yoghurt (20.12%) when kept for 2 h under room temperature on 15th day of storage. It indicates that the agar incorporated yoghurt has a better consistency compared to gelatin containing set yoghurt.

Proximate composition

Proximate composition of developed yoghurt; Protein: 3.40, Fat: 3.10, Moisture: 77.34, Ash: 0.75 (Table 9) was within the recommended range and additionally agar incorporated yoghurt contained 0.18% of dietary fiber.

Discussion and Conclusion

Agar extracted from Ceylon moss (*G. verrucosa*) using the developed method has properties (Gel strength, setting temperature, melting temperature, ash content) compatible with the international standards for agar. Using 1 kg of dried *G. verrucosa* plants, 380 g of agar powder yield can be obtained using the developed method. To prepare 1 L of yoghurt, 2.5 g of agar powder is required. Agar incorporated set yoghurt has sensory attributes similar to gelatin yoghurt and it has shelf life of 16 days under refrigerated conditions. It may have a high consumer demand in local market according to the results of sensory evaluation and it would fulfill the demand for dairy products among local vegetarian community and Muslim community who pay attention on whether the gelatin added to the yoghurt is Halal certified or not.

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