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PROCESS FOR ENHANCING THE VIABLE COUNTS OF LACTIC ACID BACTERIA AND USEFUL COMPOSITIONS THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a PCT filing for US provisional application No. 62043599 and 62063453 filed on 29 August 2014 and 14 October 2014 respectively.

BACKGROUND OF THE INVENTION

[Para0001] FIELD OF THE INVENTION

[Para0002] The invention in general relates to *Bacillus coagulans* (Lactic acid bacteria). More specifically, the invention relates to (i) the growth promotional activity of natural plant based fibers on *Bacillus coagulans* MTCC 5856; (ii) The production of short chain fatty acids (SCFA) by *Bacillus coagulans* MTCC 5856 using plant based natural fibers; and (iii) the combination of natural plant based fibers and *Bacillus coagulans* MTCC 5856 to inhibit Gram negative pathogenic bacteria.

[Para0003] DESCRIPTION OF PRIOR ART

[Para0004] Combining multistrain probiotics (probiotic bacteria) and prebiotics to achieve enhanced immunosupportive effects is well known in the art. Specifically combining probiotics with natural plant based fibers to formulate synbiotics is reported as a promising therapeutic approach (Stig Bengmark and Robert Martindale. "Prebiotics and Synbiotics in Clinical Medicine". Nutr Clin Pract vol 20 244–261, April 2005). The success of such an approach depends on carefully choosing specific probiotic microorganisms whose viable count is effectively enhanced by natural plant based fibers that are resistant to both enzymatic and acid hydrolysis in the gut. These studies are critically important to accommodate the performance of host animals exposed to symbiotic diet regimes given the teaching that there

are limitations to fiber digestion and utilisation by microbes in terms of microbial accessibility to substrates, physical and chemical nature of fibers (forage) and also kinetics of the digestive process (Gabriella A. Varga and Eric S. Kolver, "Microbial and Animal Limitations to Fiber Digestion and Utilization", J. Nutr. May 1, 1997 vol. 127 no. 5 819S-823S).

[**Para0005**] It is the principle objective of the present invention to evaluate the performance of selected natural fibers (enzyme and acid hydrolysis resistant) to enhance the viable counts of *Bacillus coagulans* MTCC 5856.

[**Para0006**] It is also another objective of the present invention to evaluate the ability of the synbiotic composition (natural fibers and *Bacillus coagulans* MTCC 5856) to inhibit pathogenic Gram negative bacteria.

[**Para0007**] It is yet another objective of the present invention to evaluate the ability of the synbiotic composition (natural fibers and *Bacillus coagulans* MTCC 5856) to produce desired short chain fatty acids, said property having profound therapeutic applications.

[**Para0008**] The present invention fulfils the aforesaid objectives and provides further related advantages.

SUMMARY OF THE INVENTION

[**Para0009**] Disclosed is (i) the growth promotional activity of natural plant based fibers on *Bacillus coagulans* MTCC 5856; (ii) the combination of natural plant based fibers and *Bacillus coagulans* MTCC 5856 to inhibit Gram negative pathogenic bacteria and (iii) the production of short chain fatty acids (SCFA) by *Bacillus coagulans* MTCC 5856 using plant based natural fibers.

[**Para0010**] Other features and advantages of the present invention will become apparent from the following more detailed description, taken in conjunction with the accompanying images, which illustrate, by way of example, the principle of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[Para0011] Figs.1a, 1b and 1c show the graphical representation of the increase in viable colony count of *Bacillus coagulans* MTCC 5856 in presence of different natural plant fibers alone (%, w/v).

[Para0012] Figs. 2a, 2b and 2c show the graphical representation of the increase in viable colony count of *Bacillus coagulans* MTCC 5856 in presence of different natural plant fibers (%, w/v) in MRS media (devoid of dextrose) (0.5, 1.0, 2.0%, w/v).

[Para0013] Fig.3 is the graphical representation of inhibition of *E. coli* ATCC 25922 growth by *B. coagulans* MTCC 5856 when co-cultured in plant based natural fibers as media. Average mean of viable counts are expressed in $\log_{10}$ cfu/ml.

[Para0014] Fig.4 shows the production of total short chain fatty acid (acetate, butyrate, and propionate) by the *B. coagulans* MTCC 5856 from Fenugreek seed fibers (FSF), *Lycium barbarum* seed fibers (LSF), Flax seed fibers (FLSF), Coconut Fibers (CF), Ginger rhizome fibers (GRF), Amla Fruit Fiber (Soluble + Insoluble) (AFF), Amla Soluble Fibers (ASF), Amla Insoluble Fibers (AIF), Psyllium husk Fibers (PHF), Cranberry seed Fibers (CSF), Fructooligosaccharide (FOS), MRS Media (MRS), and MRS Media devoid of Dextrose (MRSD). Average mean of the SCFAs are expressed in mg per gram of fiber.

DESCRIPTION OF THE MOST PREFERRED EMBODIMENTS

[Para0015] In the most preferred embodiment, the present invention relates to a method of increasing the viable colony count of *Bacillus coagulans* MTCC 5856 said method

comprising step of growing *Bacillus coagulans* MTCC 5856 in the presence of natural plant fibers selected from the group consisting of *Trigonella foenum-graecum (fenugreek) seed fibers, Lycium barbarum seed fibers, Linum usitatissimum (Flax) seed fibers, Cocos nucifera (Coconut) fibers, Zingiber officinale (Ginger) rhizome fibers, Emblica officinalis (Amla) fruit fibers, Plantago ovata (Psyllium) fibers and Vaccinium oxycoccos (Cranberry) seed fibers.*

[Para0016] In another most preferred embodiment, the present invention relates to a method of inhibiting pathogenic Gram negative bacteria said method comprising step of bringing to contact *said* Gram negative bacteria with *Bacillus coagulans* MTCC 5856 co-cultured with natural plant fibers selected from the group consisting of *Trigonella foenum-graecum (fenugreek) seed fibers, Lycium barbarum seed fibers, Linum usitatissimum (Flax) seed fibers, Cocos nucifera (Coconut) fibers, Zingiber officinale (Ginger) rhizome fibers, Emblica officinalis (Amla) fruit fibers, Plantago ovata (Psyllium) fibers and Vaccinium oxycoccos (Cranberry) seed fibers.*

[Para0017] In yet another most preferred embodiment, the present invention also relates to a method of producing short chain fatty acids by co-culturing *Bacillus coagulans* MTCC 5856 with natural plant fibers selected from the group consisting of *Trigonella foenum-graecum* (fenugreek) seed fibers, *Lycium barbarum* seed fibers, *Linum usitatissimum* (Flax) seed fibers, *Cocos nucifera* (Coconut) fibers, *Zingiber officinale* (Ginger) rhizome fibers, *Emblica officinalis* (Amla) fruit fibers, *Plantago ovata* (Psyllium) fibers and *Vaccinium oxycoccos* (Cranberry) seed fibers. In alternate embodiments, the present invention also relates to a method of protecting against diet-induced obesity and insulin resistance in the mammalian gut by administering composition comprising *Bacillus coagulans* MTCC *5856* with natural plant fibers selected from the group consisting of *Trigonella foenum-graecum* (fenugreek) seed fibers, *Lycium barbarum* seed fibers, *Linum usitatissimum* (Flax) seed fibers, *Cocos nucifera* (Coconut) fibers, *Zingiber officinale* (Ginger) rhizome fibers, *Emblica officinalis*

(Amla) fruit fibers, *Plantago ovata* (Psyllium) fibers and *Vaccinium oxycoccos* (Cranberry) seed fibers to bring about the effect of protection against diet induced obesity and insulin resistance.

[Para0018] The specific examples included herein below illustrate the aforesaid most preferred embodiments of the present invention.

[Para0019] Example I

[Para0020] Method of preparing fibers

[Para0021] ***Trigonella foenum-graecum*** **(Fenugreek) seed fibers**

[Para0022] *Trigonella foenum-graecum* (also known as Fenugreek) seeds were collected from local market and milled to course powder (10 mesh pass through). Further, four volumes of n-hexane was added to 100 gm of *Trigonella foenum-graecum* seeds course powder and extracted at reflux temperature. n-Hexane fraction was filtered through Whatman filter no 1 and again three times same extraction was carried out. After extraction, retentate was dried at 80°C for 5 h and then this was milled to obtain 40 mesh pass through powder. In an alternative method, fat or oil from Fenugreek seeds were removed by Super Critical fluid extraction method using liquid CO_2 as solvent. To increase the dietary fiber (Galactomannans) content, the enzymatic hydrolysis using Cellulase was carried out. The galactomannans content was determined by Megazyme kit (K-GALM 03/11) as per the manufacturer's instructions (Megazyme International Ireland, Bray Business Park, Bray, Co. Wicklow, IRELAND).

[Para0023] ***Lycium barbarum*** **seed fibers**

[Para0024] Goji, Goji berry or Wolfberry is the fruit of *Lycium barbarum*. The fruit of *Lycium barbarum* was dried and seeds were separated and milled to course powder. Further,

four volumes of n-hexane was added to 100 gm of *Lycium barbarum* course powder and extracted at reflux temperature. n-Hexane fraction was filtered through Whatman filter no 1 and again three times same extraction was carried out. After extraction, retentate was dried at 80°C for 5 h and then this was milled to obtain 60 mesh pass through powder. Total dietary fiber was determined by Enzymatic-Gravimetric Method (AOAC 985.29).

[Para0025] *Linum usitatissimum* (Flax seed) fibers

[Para0026] *Linum usitatissimum* (also known as common flax or linseed or Flax) seeds were collected and milled to course powder (10 mesh pass through). Further, four volumes of n-hexane was added to 100 gm of *Linum usitatissimum* course powder and extracted at reflux temperature. n-Hexane fraction was filtered through Whatman filter no 1 and again three times same extraction was carried out. After extraction, retentate was dried at 80°C for 5 h and then this was milled to obtain 40 mesh pass through powder. Total dietary fiber was determined by Enzymatic-Gravimetric Method (AOAC 985.29).

[Para0027] *Cocos nucifera* fibers

[Para0028] A matured coconut was procured from local market and dried. Further, the endosperm (coconut meat) was chopped to course and uniform size material. Further, four volumes of n-hexane was added to 100 gm of *Cocos nucifera* course material and extracted at reflux temperature. n-Hexane fraction was filtered through Whatman filter no 1 and again three times same extraction was carried out. After extraction, retentate was dried at 80°C for 5 h and then this was milled to obtain 60 mesh pass through powder. Total dietary fiber was determined by Enzymatic-Gravimetric Method (AOAC 985.29).

[Para0029] *Zingiber officinale* (ginger) rhizome fibers

[Para0030] *Zingiber officinale* rhizome, ginger root or simply ginger was dried and milled to course powder (10 mesh pass through). Further, four volumes of n-hexane was added to 100 gm of *Zingiber officinale* rhizome course powder and extracted at reflux temperature. n-Hexane fraction was filtered through Whatman filter no 1 and again three times same extraction was carried out. In an alternative method, fat or oil from Fenugreek seeds were removed by Super Critical fluid extraction method using liquid CO_2 as solvent. After extraction, retentate was dried at 80°C for 5 h and then this was milled to obtain 40 mesh pass through powder. Total dietary fiber was determined by Enzymatic-Gravimetric Method (AOAC 985.29).

[Para0031] *Emblica officinalis* (Amla) fruit fibers

[Para0032] *Emblica officinalis (Phyllanthus emblica)*, also known as Emblic, Emblic myrobalan, Myrobalan, Indian gooseberry, Malacca tree, or Amla from Sanskrit Amalika. The fruit of *Emblica officinalis* was procured from local market and dried, pulverized and passed through 60 mesh. This powder was used for the extraction of fibers. Total dietary fiber was determined by Enzymatic-Gravimetric Method (AOAC 985.29).

[Para0033] Example 2-Acid Hydrolysis

[Para0034] Two grams of plant based natural fibers listed in **Table 1** were dissolved in 100 ml of HCl (0.10 M) and incubated at 37°C with 100 rpm for 180 min. Samples were taken at 0, 30, 60, 90, 120 and 180 min. Fructooligosaccharide (FOS; Tata Chemicals, India) was also taken in the study as reference to compare with natural fibers and starch (Potato soluble starch; HiMedia, Mumbai, India) was also taken as control. The increase in reducing

carbohydrates was measured by Dinitrosalicylate reagent (Nilsson and Bjorck 1988. Journal of Nutrition 118, 1482–1486).

Table 1

Total dietary fiber content of plant based natural fibers

S. No.	Natural Fibers	Dietary Fibers (%, w/w)		
		Soluble Fibers	**Insoluble Fibers**	**Total**
1	*Trigonella foenum-graecum* (Fenugreek) seed fibers	65.42 ±1.2	5.41±0.4	75.24±1.2
2	*Lycium barbarum* seed fibers	ND	38.05±0.9	39.71±1.1
3	*Linum usitatissimum* (Flax) seed fibers	34.68±0.4	12.73±0.1	50.21±1.5
4	*Cocos nucifera* (Coconut) fibers	33.16±0.7	36.16±0.2	65.41±1.7
5	*Zingiber officinale* (Ginger) rhizome fibers	ND	41.55±0.7	42.18±0.8
6	*Emblica officinalis* (Amla) fruit fibers	10.15±0.2	41.58±0.5	52.07±1.4
	- Soluble Fraction	19.04±0.5	1.527±0.1	22.29±0.4
	- Insoluble Fraction	27.05±0.7	3.55±0.1	33.03±0.7
7	*Plantago ovata* (Psyllium) Fibers	51.13±0.8	30.24±0.8	83.24±1.5
8	*Vaccinium oxycoccos* (Cranberry) seed fibers	10.21±0.6	39.81±07	51.92±1.3

ND, Not detected; Total dietary fiber was determined by Enzymatic-Gravimetric Method (AOAC 985.29).

[Para0035] Table 2 shows the effect of acid hydrolysis on (0.1 M HCl; 37°C, 100 rpm) on Plant Based Natural Fibers. Total reducing sugar was determined by Dinitrosalicylic acid (DNSA) method.

Table 2

S.No.	Plant Based Natural Fibers	Percentage of Total Reducing Sugar					
		0 min	30 min	60 min	90 min	120 min	180 min
1	Fenugreek seed fibers	3.79±0.1	3.90±0.1	3.95±0.09	3.33±0.1	4.22±0.2	4.45±0.1
2	*Lycium barbarum* seed fibers	11.91±0.2	12.05±0.2	12.98±0.3	11.94±0.4	12.98±0.3	12.51±0.2
3	Flax seed fibers	1.24±0.1	1.89±0.09	1.97±0.1	2.10±0.1	1.98±0.1	2.21±0.1
4	Coconut Fiber	6.11±0.09	6.51±0.2	7.25±0.1	8.35±0.2	9.95±0.2	10.05±0.5
5	Ginger rhizome fibers	4.02±0.1	7.24±0.5	7.98±0.3	8.1±0.7	7.8±0.2	7.5±0.3
6	Amla Fruit Fiber (Soluble + Insoluble)	16.59±0.2	16.14±0.7	16.84±0.3	15.16±0.4	17.12±0.6	16.98±0.6
7	Amla Soluble Fiber	19.96±0.3	24.75±0.5	24.13±0.6	23.42±0.7	22.76±0.8	23.17±0.5
8	Amla Insoluble Fiber	6.98±0.1	6.74±0.1	6.92±0.2	6.95±0.2	7.18±0.1	7.60±0.1
9	Psyllium husk Fiber	0.62±0.01	1.11±0.09	1.28±0.04	1.42±0.1	1.51±0.1	1.62±0.1
10	Cranberry seed Fiber	19.70±0.4	19.15±0.3	20.50±0.9	19.76±0.7	19.67±0.9	20.60±0.8
11	Fructooligosaccharide (FOS)	1.48±0.1	6.29±0.1	8.96±0.1	9.63±0.2	10.47±0.2	12.52±0.4
12	Potato Soluble Starch	7.60±0.2	21.43±0.2	21.03±0.5	25.05±0.7	27.11±0.7	34.20±0.3

[Para0036] Example 3-Enzymatic hydrolysis

[Para0037] 100 mg of Pancreatin from Porcine pancreas 4 × USP (Sigma-Aldrich Corporation St. Louis MO, USA) was dissolved in 100 ml of phosphate buffer (50 mM; pH 7.0). Further, plant based natural fibers (2 gm) were dissolved in above Pancreatin solution and incubated at 37°C with 100 rpm for 180 min. Samples were taken at 0, 30, 60, 90, 120 and 180 min. FOS was also taken in the study as reference to compare with plant based natural fibers and starch was also taken as control. The increase in reducing carbohydrates was measured with a Dinitrosalicylate reagent (Oku et al. 1984. Journal of Nutrition 114,

1574–1581). The effect of enzymatic hydrolysis (0.1% Pancreatin in 20mM PBS pH 7.0; 37°C, 100 rpm) on plant based natural fibers is represented in **Table 3**. Total reducing sugar was determined by Dinitrosalicylic acid (DNSA) method.

Table 3

S. No.	Plant Based Natural Fibers	Percentage of total reducing sugar					
		0 min	30 min	60 min	90 min	120 min	180 min
1	Fenugreek seed fibers	7.20±0.1	8.15±0.1	11.85±0.8	11.35±0.8	10.55±0.1	10.70±0.2
2	*Lycium barbarum* seed fibers	11.86±0.2	18.11±0.8	17.85±0.2	17.75±0.4	17.53±0.8	18.21±0.4
3	Flax seed fibers	1.12±0.1	2.56±0.05	2.57±0.1	2.98±0.1	2.78±0.08	2.88±0.04
4	Coconut Fiber	8.85±0.2	11.65±0.2	14.25±0.5	13.60±0.2	10.90±0.4	11.05±0.08
5	Ginger rhizome fibers	4.14±0.09	6.94±0.2	7.12±0.3	7.31±0.4	6.81±0.1	6.82±0.09
6	Amla Fruit Fibers (Soluble + Insoluble)	16.05±0.6	16.94±0.2	16.48±0.2	15.96±0.2	17.40±0.5	16.53±0.7
7	Amla Soluble Fibers	20.10±0.7	22.18±0.8	20.51±0.4	20.90±0.8	23.72±0.8	23.07±0.5
8	Amla Insoluble Fibers	7.12±0.1	7.58±0.3	7.94±0.6	8.19±0.1	8.38±0.01	8.45±0.1
9	Psyllium husk Fiber	1.05±0.1	2.21±0.1	2.26±0.1	2.28±0.02	2.29±0.05	3.07±0.1
10	Cranberry seed Fiber	19.74±0.9	21.99±0.8	24.33±0.3	23.95±0.4	23.58±0.4	23.85±0.7
11	Fructooligosaccharide (FOS)	1.10±0.01	3.37±0.09	3.12±0.09	3.01±0.1	3.43±0.09	3.34±0.08
12	Potato Soluble Starch	7.45±0.05	52.56±0.9	54.06±1.1	52.29±1.2	52.10±1.5	54.52±1.1

[Para0038] Example 4-Growth Promotional activity of Plant Based Natural Fibers with *Bacillus coagulans* MTCC 5856

[Para0039] Single isolated colony of *Bacillus coagulans* MTCC 5856 was inoculated into MRS broth (pH 7.0±20; Himedia, Mumbai, India) and incubated at 37°C with 120 rpm for

overnight. Plant based natural fibers alone (0.5, 1.0, 2.0%, w/v), and in MRS media (devoid of dextrose) (0.5, 1.0, 2.0%, w/v) were prepared. MRS broth and MRS (devoid of dextrose) were also prepared separately. Similarly, Fructooligosaccharide (FOS) was also taken in the study as reference control to compare with plant based natural fibers. The final pH of all the media was adjusted to 7.0. Five percent of overnight grown *Bacillus coagulans* MTCC 5856 culture was inoculated to all the flasks and incubated at 37°C with 100 rpm for 24 h. pH values at 0 h of incubation and after fermentation (24 h) were also recorded. Samples were serially diluted in sterile saline and the viable count was enumerated by plating on glucose yeast extract agar (HiMedia, Mumbai, India) at 0 h and after fermentation (24 h). The plates were incubated at 37°C for 48 to 72 h. Each analysis was performed in triplicate at two different occasions. Average mean of viable counts are expressed in $\log_{10}$ cfu/ml **(Figs 1a, 1b and 1c).**

[Para0040] Example 5-Inhibition of *E. coli* growth

[Para0041] The in-vitro experiment was designed to evaluate the effect of Plant based natural fibers with probiotic bacteria *Bacillus coagulans* MTCC 5856 for the inhibition of Gram negative pathogenic bacteria *E. coli*. Briefly, 2.0 g of plant based natural fibers were added to 100 ml of demineralized water. Psyllium husk Fiber and Flax seed fibers were added 0.5 gm to 100 mL of demineralized water due to high gelling property. The pH was adjusted to 7.0±0.2 and autoclaved at 121°C for 20 min. After sterilization, oxygen reducing enzyme Oxyrase (Oxyrase® for Broth, Oxyrase, Inc, Mansfield, OH, USA) was added to each flask. Bacillus coagulans MTCC 5856 was grown on glucose yeast extract agar (Himedia, Mumbai, India) and *E. coli* ATCC 25922 was grown on trypticase soya agar (Himedia, Mumbai, India). Single isolated colony of both the cultures was used and the turbidity of the bacterial suspension was adjusted to 0.5 McFarland standards (equivalent to 1.5 × 108 colony forming units (CFU)/ml). One milliliter of *E. coli* ATCC 25922 was added to flask containing plant

based natural fiber. Similarly, in other group 1 ml of *E. coli* ATCC 25922 and 1 ml of *B. coagulans* MTCC 5856 were added to flask containing plant based natural fiber. The flasks were incubated at 37°C with 100 rpm for 24 h. Samples were serially diluted in sterile saline and the viable count of E. coli ATCC 25922 was enumerated by plating on Eosin Methylene Blue Agar (EMB Agar; HiMedia, Mumbai, India) at 0 h and after fermentation (24 h). The plates were incubated at 37°C for 48 h. Each analysis was performed in triplicate at two different occasions. Average mean of viable counts are expressed in $\log_{10}$ cfu/ml (**Fig.3**).

[Para0042] Example 6-Production of SCFA by *Bacillus coagulans* MTCC 5856 using Plant Based Natural Fibers

[Para0043] The in vitro fermentation with the *Bacillus coagulans* MTCC 5856 was carried out by following method described by McBurney and Thompson (McBurney MI and Thompson LU. (1987) Effect of human faecal inoculum on in vitro fermentation variables. Brit J Nutr 58: 233-243) with some modifications. Briefly, 2.0 g of glucose or Plant Based Natural Fibers were added to 100 mL of demineralised water. Psyllium husk Fiber and Flax seed fibers were added 0.5 gm to 100 ml of demineralised water due to high gelling property. The pH was adjusted to 7.0±0.2 and autoclaved at 121°C for 20 min. After sterilization, oxygen reducing enzyme Oxyrase (Oxyrase® for Broth, Oxyrase, Inc, Mansfield, OH, USA) was added to each flask, to induce anaerobic conditions. Five percent of overnight grown *Bacillus coagulans* MTCC 5856 culture was inoculated to all the flasks and incubated at 37°C with gentle shaken rpm for 24 h. The bottles were tightly closed and sealed with parafilm to maintain anaerobic conditions generated by the enzyme supplement. pH values at 0 h of incubation and after fermentation (24 h) were also recorded. One ml of copper sulphate (10g/L) was added to each sample to inhibit further microbial growth (Sigma, St. Louis, MO, USA). The analysis of short chain fatty acids in the aforesaid fermentation samples was done adopting the following parameters.

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Reagents

1. Diethyl Ether (AR Grade)
2. H_2SO_4
3. RO Water
4. Sodium Chloride (AR Grade)

Chromatographic conditions

Oven:

Rate	Temperature	Hold time
Initial	80 ° C	1.00 minute
8° C/minute	200° C	2.00 minute

1. Post run temperature 220^0C
2. Post Time 5.0 min
3. Run time 18.00 min

Inlet

Injection Volume	1 µl
Temperature	250^0C
Mode	Split
Split ratio	10:1

Column

1. DB-FFAP (Terephthalic acid modified poly ethylene Glycol)
2. Dimensions: 30.00m X 250.00 mm X 0.25 µm.
3. Carrier gas : Nitrogen

4. Flow: 1.0 ml / min

Detector

1.	Type	FID
2.	Temperature	350^0C
3.	Hydrogen Flow	30.0ml/min
4.	Air flow	300.0 ml/min
5.	Make Up Flow	5.0 ml/min

[Para0044] Standard solution preparation

[Para0045] 100.0 mg of each of Fatty acid standard (Acetic acid, Propionic acid and Butyric acid) was weighed accurately in a 100ml volumetric flask & dissolved in 50.0 mL of water and made up to the mark with water and mixed well (Stock solution). Further, 10.0 mL of the stock solution was diluted to 100.0 mL with water and mixed well to get standard solution. 5 mL of standard solution was subjected to extraction as described herein below.

1. Taken 5ml of Standard solution / sample.
2. To Standard solution added 5ml of water with vortexing for 0.5 min.
3. Adjusted pH to 1-1.5 with 3M H_2SO_4 with vortexing for 0.5 min.
4. Kept diethyl ether in -20°C up to 1hr before adding to the sample /Working Standard.
5. Added 10ml of diethyl ether with vortexing for 1 min.
6. Added 4g of Sodium Chloride with vortexing for 1 min.
7. Centrifuged to separate Water Layer & Diethyl Layer
8. Transferred 1.0 mL of Diethyl Ether layer in GC Vial & Injected.

Procedure:

1 µl each of extracted standard solution was injected into the chromatograph in triplicate and recorded the responses of major peaks due to Acetic acid, Propionic acid and Butyric acid. The % Relative Standard Deviation for area of peaks due to Acetic acid, Propionic acid and Butyric acid in triplet injections should not be more than 2.0 %. Injected 1.0 µl each of extracted sample solution into the chromatograph. The content of Acetic acid, Propionic acid and Butyric acid was calculated as follows.

$$\text{Content of individual acid in ppm} = \frac{\text{Sample area x Std. conc. (ppm)}}{\text{Standard area}}$$

[Para0046] The results of the chromatographic analysis are presented in **Fig 4** and represented herein below as Tables 4 and 5.

In Table 4 it may be noted that the production (mg/gram of fiber) of short chain fatty acid (Acetate, Propionate and Butyrate) was from plant based natural fibers as a sole nutritional source in vitro batch –culture fermentation with *B. coagulans* MTCC 5856. FOS was used as reference control in the study.

In Table 5 it may be noted that the production (mg/gram if fiber) of short chain fatty acid (acetate, propionate and butyrate) was from plant based natural fibers along with other nutrients in vitro batch –culture fermentation with *B. coagulans* MTCC 5856. In MRS media dextrose was replaced by plant based natural fibers for the production of SCFA. FOS was used as reference control in the study. MRS media and Media devoid of Dextrose (MRSD) were also taken to compare for the production of the study.

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Table 4

S. No.	Plant Based Natural Fibers (alone)	Short Chain Fatty Acids (mg/gram of Fibers)		
		Acetate	**Butyrate**	**Propionate**
1	Fenugreek seed fibers	69.79	3.36	0.27
2	*Lycium barbarum* seed fibers	77.18	6.56	0.24
3	Flax seed fibers	109.5	5.93	0.27
4	Coconut Fibers	49.39	0.96	0.14
5	Ginger rhizome fibers	1.62	0.20	42.35
6	Amla Fruit Fiber (Soluble + Insoluble)	3.87	0.30	79.99
7	Amla Soluble Fibers	5.50	0.23	76.55
8	Amla Insoluble Fibers	1.05	0.25	44.10
9	Psyllium husk Fibers	1.70	0.14	38.11
10	Cranberry seed Fibers	8.64	0.225	69.07
11	Fructooligosaccharide (FOS)	1.07	0.07	21.61

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Table 5

S. No.	Plant Based Natural Fibers along with MRS Media devoid of dextrose	Short Chain Fatty Acids (mg/gram of Fibers)		
		Acetate	**Propionate**	**Butyrate**
1	Fenugreek seed fibers	84.67	3.68	0.97
2	*Lycium barbarum* seed fibers	61.05	5.41	0.75
3	Flax seed fibers	146.86	7.52	1.43
4	Coconut Fibers	51.91	1.01	0.51
5	Ginger rhizome fibers	118.72	7.90	1.32
6	Amla Fruit Fiber (Soluble + Insoluble)	94.11	6.46	1.06
7	Amla Soluble Fibers	90.99	9.37	1.24
8	Amla Insoluble Fibers	79.10	1.70	0.38
9	Psyllium husk Fibers	53.24	3.23	0.56
10	Cranberry seed Fibers	110.89	9.58	1.69
11	Fructooligosaccharide (FOS)	73.96	4.30	0.24
12	MRS Media	113.07	1.04	0.05
13	MRS Media devoid of Dextrose (MRSD)	50.36	1.17	0.16

[Para0047] While the invention has been described with reference to a preferred embodiment, it is to be clearly understood by those skilled in the art that the invention is not limited thereto. Rather, the scope of the invention is to be interpreted only in conjunction with the appended claims.

~~AMENDMENT TO THE CLAIMS~~

~~REPLACEMENT SHEET~~

We claim,

1. A method of increasing the viable colony count of *Bacillus coagulans* MTCC 5856 said method comprising step of growing *Bacillus coagulans* MTCC 5856 in the presence of natural plant fibres selected from the group consisting of *Trigonella foenum-graecum* (fenugreek) seed fibers, *Lycium barbarum* seed fibers, *Linum usitatissimum* (Flax) seed fibers, *Cocos nucifera* (Coconut) fibers, *Zingiber officinale* (Ginger) rhizome fibers, *Emblica officinalis* (Amla) fruit fibers, *Plantago ovata* (Psyllium) fibers and *Vaccinium oxycoccos* (Cranberry) seed fibers.
2. A method of inhibiting pathogenic Gram Negative bacteria said method comprising step of bringing to contact said Gram Negative bacteria with *Bacillus coagulans* MTCC 5856 co-cultured with natural plant fibres selected from the group consisting of *Trigonella foenum-graecum* (fenugreek) seed fibers, *Lycium barbarum* seed fibers, *Linum usitatissimum* (Flax) seed fibers, *Cocos nucifera* (Coconut) fibers, *Zingiber officinale* (Ginger) rhizome fibers, *Emblica officinalis* (Amla) fruit fibers, *Plantago ovata* (Psyllium) fibers and *Vaccinium oxycoccos* (Cranberry) seed fibers.
3. A method of producing short chain fatty acids by co-culturing *Bacillus coagulans* MTCC 5856 with natural plant fibres selected from the group consisting of *Trigonella foenum-graecum* (fenugreek) seed fibers, *Lycium barbarum* seed fibers, *Linum usitatissimum* (Flax) seed fibers, *Cocos nucifera* (Coconut) fibers, *Zingiber officinale* (Ginger) rhizome fibers, *Emblica officinalis* (Amla) fruit fibers, *Plantago ovata* (Psyllium) fibers and *Vaccinium oxycoccos* (Cranberry) seed fibers.
4. A method of protecting against diet-induced obesity and insulin resistance in the mammalian gut by administering composition comprising *Bacillus coagulans* MTCC

5856 with natural plant fibres selected from the group consisting of *Trigonella foenum-graecum* (fenugreek) seed fibers, *Lycium barbarum* seed fibers, *Linum usitatissimum* (Flax) seed fibers, *Cocos nucifera* (Coconut) fibers, *Zingiber officinale* (Ginger) rhizome fibers, *Emblica officinalis* (Amla) fruit fibers, *Plantago ovata* (Psyllium) fibers and *Vaccinium oxycoccos* (Cranberry) seed fibres to bring about the effect of protection against diet induced obesity and insulin resistance.

5. Compound comprising Bacillus coagulans MTCC 5856 with natural plant fibres selected from the group consisting of Trigonella foenum-graecum (fenugreek) seed fibers, Lycium barbarum seed fibers, Linum usitatissimum (Flax) seed fibers, Cocos nucifera (Coconut) fibers, Zingiber officinale (Ginger) rhizome fibers, Emblica officinalis (Amla) fruit fibers, Plantago ovata (Psyllium) fibers and Vaccinium oxycoccos (Cranberry) seed fibres for use in protection against diet-induced obesity and insulin resistance.

6. The use of compound comprising Bacillus coagulans MTCC 5856 with natural plant fibres selected from the group consisting of Trigonella foenum-graecum (fenugreek) seed fibers, Lycium barbarum seed fibers, Linum usitatissimum (Flax) seed fibers, Cocos nucifera (Coconut) fibers, Zingiber officinale (Ginger) rhizome fibers, Emblica officinalis (Amla) fruit fibers, Plantago ovata (Psyllium) fibers and Vaccinium oxycoccos (Cranberry) seed fibres in the manufacture of a medicament for protecting against diet-induced obesity and insulin resistance.