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Characterization of custard apple seeds for the separation of prebiotic low molecular weight carbohydrates (LMWC)

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Abstract

The concept of waste to wealth is gaining lot of importance in the recent days. In this context, the present study was undertaken to identify the potential prebiotics present in the custard apple seeds. Among the different components identified, the Low Molecular Weight Carbohydrates (LMWC) were chosen for the study and the extraction was performed using Ethanol at three different concentrations (50%, 75% and 95%) and different combination of temperature, time and speed of rotation. The maximum yield of 9.16% was obtained with 50% Ethanol, 37 °C, 72h and 60 rpm. The probiotic organism *Lactobacillus bulgaricus* displayed a positive growth of 7.54 ± 0.04 log cfu/ml on LMWC over a period of 24 hours which confirmed the prebiotic functionality of LMWC. Scanning Electron Microscopy and FTIR analysis were done to confirm the morphological characteristics. Therefore, all the results obtained showed that LMWC can be used as potential prebiotics in the food industry and the commercial utilization of custard apple seeds.

Keywords: Custard apple, low molecular weight sugars, extraction, prebiotics

1. Introduction

The sugar apple or sweet sop (*Annona squamosa*), commonly called as Sitaphal in traditional language, and is the fruit of Annonaceae family is a native of tropical America and West Indies. The fruit is nearly spherical to oval in shape with thick rind consisting of knobby segments in which the seeds are surrounded by the pulp (Morton, 1987) [16]. The pulp of the fruit is creamy white, fragrant and sweet in taste which will be adhered to seeds forming individual segments arranged in a uniform layer around the core of the fruit. The number of seeds may vary from 20-40 depending upon the variety of the fruit. According to Indiastat (2019), the total production in India was 3,38,000 tonnes over an area of 40,000 Hectares. Out of this, significant amount of the fruits are used for preparation of variety of products such as ice cream, milk shakes and other sweets. The components which remain are the seeds and the rind of the fruit. They are considered as the rich carbon source and may pose the risk of pollution if not handled properly. The custard apple seeds are usually disposed and/or they are used as a planting material. The custard apple seeds are viewed as a rich source for the extraction of essential oil (Lokhande *et al.*, 2013) [10]. The utilization of custard apple seeds as a low cost material for the extraction of various prebiotic components is gaining a lot of importance in recent days (Pallavi *et al.*, 2017) [20].

Carbohydrates are widely distributed in a variety of plants and animals as discrete molecules of low-molecular weight or as polymers, the molecular weight of which will be more than one million in certain cases. Plants synthesize these sugars by photosynthesis from carbon dioxide and water. Simple sugars can be a potential prebiotic due to their low molecular weight which will take a fairly longer time to get digested in the body. Prebiotics are the components of the food which cannot be digested in the body that will affect the host cells beneficially by selectively stimulating the growth and/or activity of one or more number of bacteria in the colon" (Gibson & Roberfroid, 1995) [4]. The characteristics of prebiotics are – (a) resistance to hydrolysis by mammalian enzymes, gastric acidity and gastrointestinal absorption; (b) fermentation by the intestinal microorganisms; (c) stimulating the selective growth and/or activity of intestinal microflora associated with health and well-being of the human; (d)

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enhancing the absorption of minerals (Scholz-Ahrens *et al.*, 2001, 2007) [23, 22]. Chemically, these are the indigestible oligo- and polysaccharides by the human digestive system and reach the intestine to stimulate the growth of beneficial bacteria and inhibit the growth of harmful ones. *Lactobacillus* and *Bifidobacteria* that share a positive relationship with human host are considered to be beneficial and hence referred as "probiotics". Since the prebiotic components acts as energy source for probiotics and allow increase in their selective growth, they ensure a competitive advantage over the harmful bacteria like *S.aureus* and *E.coli*.

With the intention of identifying the natural or the indigenous sources for the presence of prebiotic components, the present study was taken up based on these lines. Custard Apple is considered as a wonder fruit, super fruit due to its wide range of therapeutic and medicinal properties (Jamkhande *et al.*, 2014; Jamkhande & Wattamwar, 2015) [8, 7]. The main thrust is given with an intention of making a proper use of the underutilized seasonal fruit in India which is mostly consumed as fresh and not processed. The present study was focused mainly on the utilization of the seeds which are present in a considerable number in the fruit, which are usually thrown away. The experimental study was made using the D-optimal mixture design, which is multi factor design in order to extract the Low Molecular Weight Carbohydrates from the Custard Apple seeds. The extraction was performed using ethanol at three different concentrations of 50%, 75% and 95% in order to obtain the maximum yield of LMWC. The factors used in the design are the Concentration of the ethanol, Extraction temperature, Time of extraction and the Speed of rotation (rpm) of the shaking incubator. The study was majorly focused on identifying the extent of recovery of the LMWC from the custard apple seeds so that the potential components present in the seeds can be utilized for the formulation of wide range of functional and therapeutic foods which will help in boosting the immune strength and disease resistance capacity of the human beings.

2. Materials and Methods

2.1 Materials

Fresh, matured Custard apple (*Annona squamosa*) fruits were procured from Amruthabhoomi natural and organic products Ltd. Chamarajanagar, Karnataka, India. The pulp, peel and seeds were separated manually. The seeds are then washed with water and air dried at 130°C for 15 min. The outer shells of the seeds were removed by mechanical pressure. The seeds were then made into uniform granule sized powder of 500µm using electronic pulverizer (Pulverisette 14, FRISTSCH Germany). The fine powder was then stored in air tight bags to prevent the absorption of moisture.

2.2 Proximate Analysis of the Custard Apple seed powder

The proximate analysis of the custard apple seed powder was conducted as per the AOAC approved methods. Based on the literature study and the initial trials conducted, the presence of LMWC in the seeds was confirmed and further studies were carried out.

2.3 Extraction and Purification

2.3.1 Sample preparation for extraction

The custard apple seeds were defatted in hexane (B.P 66°C – 68°C) in the solid-to-liquid ratio of 4:100 g/ml for 4h and the defatted powder was dried and kept in air tight bags until further use.

2.3.2 Extraction of LMWC

LMWC were extracted from the seeds by slight modification of the method followed by Moongngarm *et al.*, 2011 [15]. Ethanol assisted extraction was followed for the separation of LMWC from custard apple seed powder with continuous shaking in the shaking incubator. Extraction was carried out using three different concentration of ethanol at 50% v/v, 75% v/v and 95% v/v at a constant temperature of 37°C. Treatment time was increased gradually with constant interval from 24h, 36h, 48h to 72h with four different rotational speeds of 60, 120, 180 and 240 rpm.

2.3.3 Purification of extracted LMWC

The pH of the solution was adjusted to 6.8 using 1M NaOH as per the method followed by (Moongngarm *et al.*, 2011) [15]. The samples were filtered using a muslin cloth and the filtrate was obtained. The solvent in the filtrate was evaporated in a continuous rotary evaporator (IKA, RV 10 Digital) with the vacuum range of 760mm Hg at the speed of rotation of 135 rpm at a temperature of 72°C.

2.3.4 Freeze drying

The LMWC extract was freeze dried in a lyophilizer (Lark lyophilize) operating at a vacuum of 1mm Hg for 72hrs. The dried sample was stored in an air tight bottle and stored at a temperature of -18 °C until further use (White & Cakebread, 1966) [28].

2.4 Experimental Design for the extraction of LMWC

Ethanol assisted LMWC extraction was carried out using Brunswick Scientific shaking incubator with the speed ranging from 60-240 rpm. The defatted custard apple seed powder was subjected to extraction with a 1:25 (w/v) sample/solution ratio at different concentration of 50%, 75% and 95% ethanol and temperatures (30, 40, 50 and 60°C) in the time range of 24-72 hours. All experiments were conducted in triplicates and at the end of each trial, the samples were taken periodically and subjected to rotary evaporation and freeze drying and the reducing sugar content was measured by DNS method using UV-Spectrophotometer at 510nm (Pallavi *et al.*, 2017) [20].

2.5 Modelling of Ethanol-assisted LMWC extraction

Ethanol assisted LMWC extraction kinetics was fitted by non-linear regression using mass transfer mathematical model for the development of a generalized model for the extraction of LMWC from custard apple seed powder. The extraction of LMWC from Custard apple seeds involves the measurement of overall mass transfer coefficient ($K_L a/V$) across an external surface area a (m^2) at any time t and at saturation conditions. Maximum extractable LMWC % (C) was obtained by three repeated continuous extractions from the sample. However, the yield of all the extraction process was recorded and the extraction kinetics was performed using MATLAB R2019 by fitting first order equation.

$$C = C_s \{ 1 - \exp(-K_L a) t/V \} \quad (1)$$

Where,

C (g/100g) = Concentration of LMWC in the solvent at any time t

C_s (g/100g) = Concentration of LMWC in the solvent at saturation conditions

K_L (min^{-1}) = overall mass transfer coefficient at a surface of area a (m^2) and volume v (m^3)

2.6 Estimation of the Prebiotic activity of the LMWC

The Probiotic cultures selected for studying the activity were *Lactobacillus bulgaricus* isolated from yoghurt and confirmed positive through Gram's staining and hydrogen peroxide test. *Staphylococcus aureus* (NCTC 3750) was used as a negative control in the study. For the determination of prebiotic activity, cultures of *L. bulgaricus* grown on MRS broth (37°C, anaerobic) and *S. aureus* (NCTC 3750) (37°C, aerobic) grown on Nutrient broth were supplemented with 1% glucose and 1% LMWC for 24 hrs and the growth count were enumerated by using spread plate technique after appropriate serial dilutions on MRS agar for *L. bulgaricus* and Nutrient agar for *S. aureus*. The numbers of colony forming units (cfu) on the plates were enumerated using a colony counter (Lapiz colony counter).

The prebiotic activity scores of LMWC were determined according to (Huebner *et al.*, 2007). Prebiotic Activity Score (PAS) = {(probiotic log cfu/ml on the prebiotic at 24 h – probiotic log cfu/ml on prebiotic at 0 h) / (probiotic log cfu/ml on glucose at 24 h – probiotic log cfu/ml on glucose at 0 h)} – {(enteric log cfu/ml on the prebiotic at 24 h – enteric log cfu/ml on the prebiotic at 0 h) / (enteric log cfu/ml on glucose at 24 h – enteric log cfu/ml on glucose at 0 h)}.

2.7 Methods for the evaluation of Quality Parameters

1) FTIR Spectrum

Fourier Transform Infrared Spectroscopy (FT-IR) was used for characterization of custard apple seed powder and purified LMWC structure (Černá *et al.*, 2003; Shaltout *et al.*, 2011) ^[1, 24]. IR spectra were collected using FT-IR Spectrophotometer, PerkinElmer, USA. The curves were produced at the resolution of 4cm⁻¹ with 16 scans ranging from 400 – 4000 cm⁻¹.

2) Scanning electron microscopy

Morphological observations of the Custard apple seed powder and the LMWC obtained after extraction were obtained by Scanning Electron Microscopy. Microscopy was carried out with (VEGA3 TESCAN, Czech Republic) model system at 10 kV accelerating voltage with 1.00 KX magnification (Parate *et al.*, 1986) ^[21].

3) Statistical data analysis

All the experimental data was obtained in triplicates and analyzed using IBM SPSS 9.0 for analysis of variance (ANOVA).

3. Results and Discussions

3.1 Proximate composition of the custard apple seeds

Custard apple seeds were chosen for the current study to identify the potential prebiotics present in them under the perspective of waste to wealth concept. A detailed study was conducted in order to identify the composition of the Custard apple seeds. All studies were made in triplicates based on the reports available on the proximate composition of the peel and seed with respect to foreign matter, moisture content and ash (Fontana Jr & Carter, 2020; Jamkhanda & Wattamwar, 2015; Pallavi *et al.*, 2017; Shardul *et al.*, 2013) ^[2, 20, 8, 25]. The results of the proximate composition are presented in Table 1.

3.2 Extraction kinetics of LMWC

Extraction processes are governed by the mass transfer principles and the separation of LMWC is a function of: a) Extraction through direct interaction of the cell matrix with the solvent, resulting in the leaching of the compounds into

the solvent; b) Evaporation of the solvent by heating up to its boiling point retaining the extracted compound (Wichienchot *et al.*, 2011) ^[29]. In order to better understand the extraction process, kinetics of extraction was performed using a single step model. The constant used to explain the extraction process was overall mass transfer Coefficient (K_L) as given in equation 1. The results obtained are given in Figure 1.

3.3 Estimation of Prebiotic Potential of LMWC

The ability of the substrate to get metabolized effectively by the beneficial bacteria in comparison to the harmful bacteria is the major factor to identify the substrate as a prebiotic. The quantification of the Prebiotic potential of the substrates can be done by determining the Prebiotic Activity Score (Huebner *et al.*, 2007; Pallavi *et al.*, 2017) ^[20]. The LMWC fractions in the purest form are directly added as an active carbon source in the bacterial growth medium. The structural-functional relationship was elucidated to find out how they could affect the prebiotic activity when they are separated from the food matrix. The growth of bacteria on glucose was used as a standard for comparison.

From the bacterial cell growth observed (Table 3), it can be assumed that the LMWC are efficiently utilized by *L. bulgaricus* than that of *S. aureus*. The data was used to calculate the prebiotic activity score. The prebiotic Activity Score (PAS) of LMWC was 0.21. A positive Prebiotic Activity Score indicated that the substrates are effectively utilized by the probiotic than glucose and are therefore considered as potential prebiotics. A negative score would indicate that the components are being underutilized by the probiotic or are better utilized by the pathogenic strains. Even though the rate of growth of *L. bulgaricus* was much higher than that of *S. aureus*, the limited amount of available LMWC as a carbon source into the medium might have affected the duration of log phase and hence, the prebiotic activity score. Therefore, the lower yield of LMWC obtained may probably indicate the low prebiotic activity score of the LMWC.

3.4 Methods for the Evaluation of Quality Parameters

3.4.1 Structural Analysis

FTIR spectra analysis of the custard apple seed powder and the extracted LMWC from the custard apple seeds were done to identify the sugars, functional groups and to compare the yield of sugars with the raw seed powder used as a control. The wavelength of the spectrum from 950cm⁻¹ to 1200 cm⁻¹ are known as the fingerprint region of the carbohydrates since it depicts the presence of major functional groups of the monosaccharides and polysaccharides (Černá *et al.*, 2003; Shaltout *et al.*, 2011) ^[1, 24]. The carbohydrates in the sample were retained after subjecting it to extraction at different treatment conditions (Figure 2). The absorption band from 1011 to 1120 cm⁻¹ corresponds to the C-O-C glycosidic ring bond stretching, O-H bending and stretching of the C-O bond in COOH. The region was same for both the seed powder and LMWC extract. The region between 890 to 1000 cm⁻¹ represents low and moderate intensity bands of C-C skeletal vibrations. Thus, the data obtained from FTIR shows the similar pattern between both samples and corresponds to the presence of LMWC sugars.

3.4.2 Scanning Electron Microscopic Imaging

SEM images for the Custard Apple Seed Powder and the extracted LMWC was shown in Figure.3. Different morphological changes in the powder surface were seen with the increase in the magnification. Figure 3 (A), (B) and (C)

are that of the custard apple seed powder under different magnifications of X150, X700 and X1000 clearly indicated that the particles were agglomerates due to high hygroscopicity of the sugars in the seed powder. In figure 3D, 3E and 3F were the freeze dried LMWC extract under X10000, X12000 and X20,000 with more compact and

amorphous structures. The particles were found to be stiff and highly rigid morphology was seen. From the observation of the SEM, the particles were tending to be appeared as amorphous surfaces because of the binding of the particles. The results were in good agreement with the work done by (Parate *et al.*, 1986) [21].

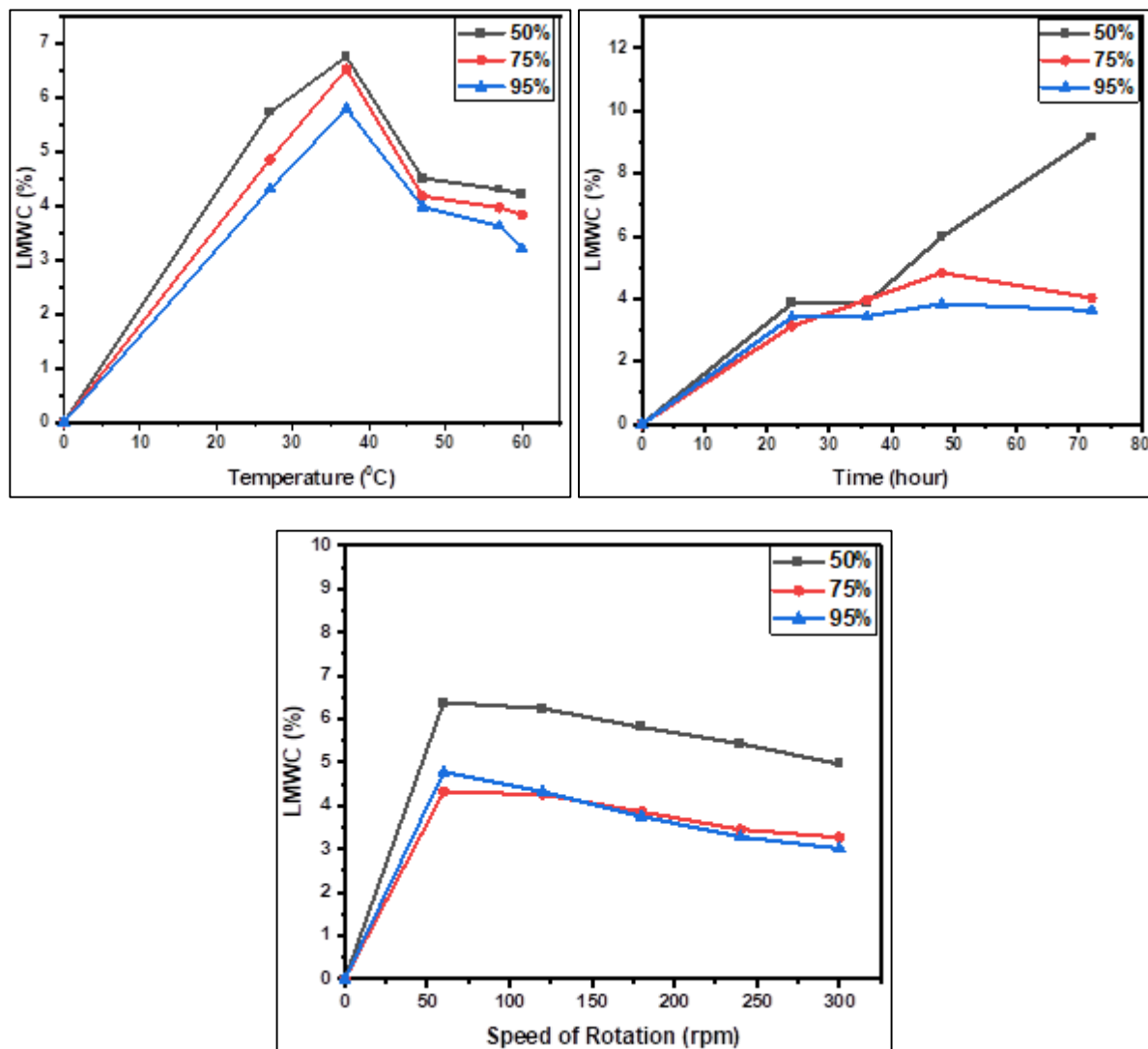


Fig 1: Effect of different treatment conditions on the yield of LMWC Temperature($^{\circ}$ C) (b) Time(h) (c) Speed of rotation(rpm)

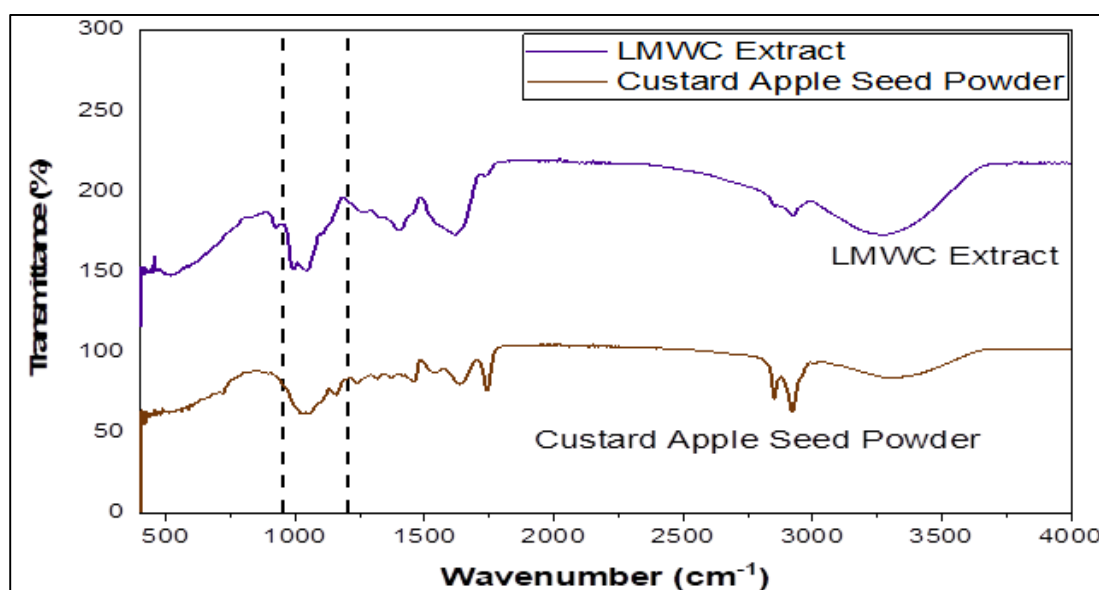


Fig 2: FTIR spectra of Custard Apple Seed powder and LMWC

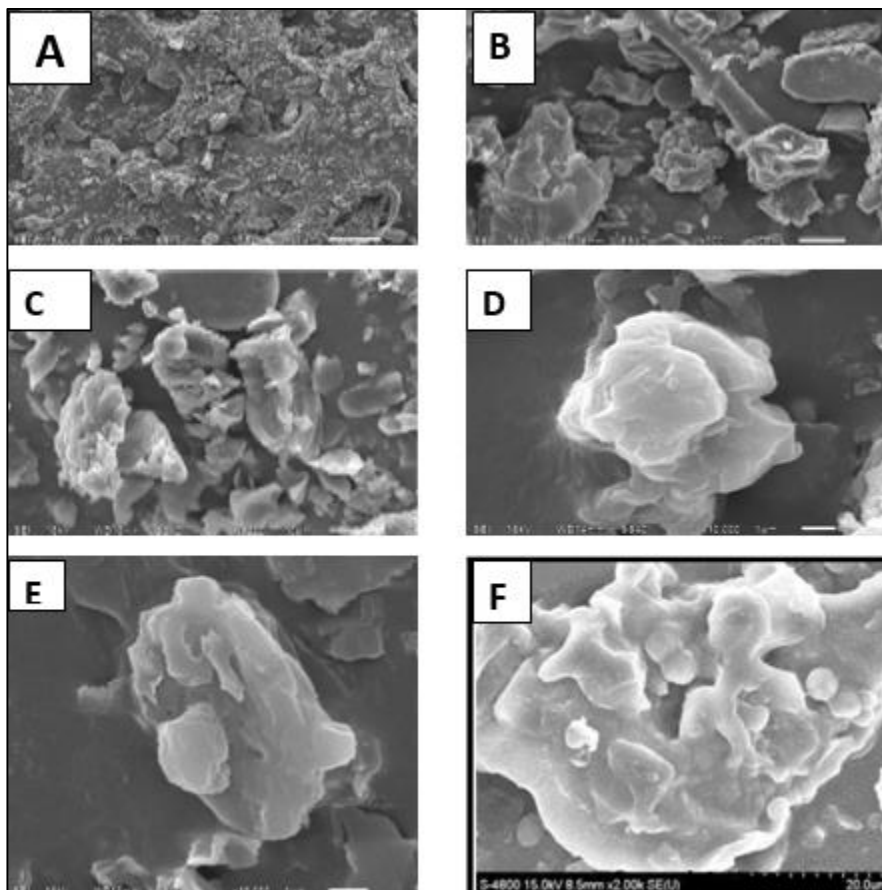


Fig 3: SEM images of Custard Apple Seed Powder under application of (A) X150 (B) X700 (C) X1000, LMWC Extract (D) X10000 (E) X12000 (F) X20000

Table 1: Physico-chemical properties of Custard Apple Seeds

Parameter	Mean±SD
Moisture (%)	28.73± 0.04
Total Fat (%)	15.17± 0.05
Crude fiber (%)	27.78± 0.06
Total Ash Content (%)	1.75± 0.15
Acid Insoluble Ash (%)	0.16± 0.08
Protein (%)	11.97±0.02
Carbohydrates (by difference) (%)	14.26± 0.06
a_w	0.26 ± 0.02
L*	52.21 ± 0.03
a*	6.58 ± 0.01
b*	20.24 ± 0.02
Swelling capacity (%)	5.0 ± 0.04
Bulk Density (g/cm ³)	0.5 ± 0.02
Water Absorption capacity (%)	201.2 ± 0.05
Oil Absorption Capacity (%)	160.95 ± 0.03
Emulsion Capacity (%)	40.00 ± 0.02
Foaming Capacity (%)	4.44 ± 0.04

Table 2: Yield of LMWC from Custard Apple seed powder under different experimental conditions

Run	Formulation Variables				Yield of LMWC (%)
	Factor 1	Factor 2	Factor 3	Factor 4	
	Ethanol (%)	Temperature (°C)	Time (h)	Speed of rotation (rpm)	
1	95	30	36	60	4.78 ± 0.02
2	75	60	48	240	3.67 ± 0.04
3	75	50	48	120	3.97 ± 0.03
4	75	30	36	240	4.83 ± 0.04
5	50	50	36	180	5.81 ± 0.02
6	50	60	72	240	4.93 ± 0.01
7	75	40	24	120	3.99 ± 0.03
8	95	40	72	120	4.53 ± 0.06
9	75	60	72	60	3.64 ± 0.03
10	75	40	48	60	4.32 ± 0.01

11	50	60	48	180	6.32 ± 0.02
12	50	50	48	60	6.38 ± 0.02
13	95	50	48	180	3.84 ± 0.03
14	95	40	48	240	3.63 ± 0.07
15	95	60	48	120	3.42 ± 0.04
16	95	30	36	180	4.12 ± 0.01
17	75	60	36	120	2.76 ± 0.03
18	75	40	36	180	3.14 ± 0.02
19	75	50	36	60	3.97 ± 0.01
20	95	50	72	60	5.23 ± 0.02
21	95	50	24	240	3.62 ± 0.02
22	75	50	24	180	3.32 ± 0.01
23	75	30	36	120	3.41 ± 0.03
24	75	60	24	180	3.79 ± 0.04
25	95	60	36	240	3.21 ± 0.05
26	50	40	36	240	5.94 ± 0.02
27	75	40	24	240	3.13 ± 0.03
28	95	50	36	120	3.74 ± 0.02
29	50	30	24	240	3.86 ± 0.04
30	50	30	48	120	4.97 ± 0.02
31	75	40	72	60	4.35 ± 0.01
32	50	30	24	180	3.12 ± 0.04
33	95	30	72	240	4.36 ± 0.05
34	50	60	36	60	3.87 ± 0.02
35	95	60	24	60	4.87 ± 0.03
36	50	40	48	180	3.98 ± 0.01
37	95	30	48	60	4.98 ± 0.02
38	75	40	72	240	3.25 ± 0.04
39	50	40	72	180	3.99 ± 0.03
40	95	40	24	180	3.64 ± 0.02
41	95	40	36	60	4.23 ± 0.04
42	75	30	48	180	3.62 ± 0.03
43	75	30	72	180	3.76 ± 0.02
44	50	30	72	60	9.16 ± 0.03
45	50	50	36	240	4.31 ± 0.04
46	95	60	72	180	4.29 ± 0.03
47	75	60	72	120	4.35 ± 0.05
48	50	30	36	120	4.32 ± 0.02
49	50	50	72	120	3.75 ± 0.03
50	50	40	36	120	3.24 ± 0.01
51	75	50	48	240	3.02 ± 0.02
52	75	30	24	60	5.03 ± 0.03
53	75	50	72	180	4.02 ± 0.02
54	50	60	24	120	3.63 ± 0.04
55	50	40	24	60	3.71 ± 0.03
56	95	30	24	120	3.01 ± 0.02
57	50	50	24	60	3.25 ± 0.04

Table 3: Bacterial Growth in the Carbohydrate Fractions of LMWC (log cfu/ml)

Carbohydrates	<i>Lactobacillus bulgaricus</i>		<i>Staphylococcus aureus</i>	
	0 h	24 h	0 h	24 h
LMWC	0	0.98±0.03	0	0.61±0.04
Glucose* (Standard)	0	1.51±0.04	0	1.08±0.04

*Growth on Glucose was used as a standard for reference

4. Conclusion

This work was done to identify and extract the components in the custard apple seeds with potential prebiotic activity. Among the different components, the low molecular weight carbohydrates (LMWC) were selected for the study and extracted using eco-friendly techniques. The yield obtained was more with 50% Ethanol, 37°C Temperature, 72 hours and 60 rpm. The positive prebiotic activity score suggested that LMWC are capable of improving the growth of potential probiotic microorganisms. Development of robust,

economical and automated methods for the utilization of Custard Apple Seeds as an ingredient in food processing and Probiotic formulations will be an added advantage both for the farmers and also the food industry. Effective utilization of the Custard Apple seeds will pave a way for the waste to wealth concept and the prebiotics extracted from the seeds can be used as an ingredient for functional foods, nutraceuticals and health foods formulations.

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