Bacteriological Study for Detection The Role of Fructan Produced from *Pseudomonas putida* as Enhancer for *Lactobacillus acidophilus* Growth

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Abstract

Fructan is a kind of biopolymer produced from plants, fungi, yeast and bacteria. It is classified as prebiotics food and enhanced the growth of probiotics bacterial strains which improves the health of the individual. This article aimed to study the production and extraction of fructan from *Pseudomonas putida* isolated from food samples and study the effect of fructan on the viable cell number of *Lactobacillus acidophilus*. The results showed that *Pseudomonas putida* have the ability to produce fructan when cultured on production medium (YPS), fructan exhibited as white powder when extracted from YPS and the amount of fructan was 3.6 mg/100 ml, also the results showed that fructan can increase the viable cell number of *Lactobacillus acidophilus* when added to MRS. FTIR analysis of fructan produced from *Pseudomonas putida* revealed that the functional groups found in chemical structure of fructan, which it was (C-O, C-H, O-H and C=O).
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Introduction

Fructan is extracellular polymers composed of D-fructofuranosyl residues linked as either β-(2, 6) or β-(2, 1), the building units of fructan is fructose residues, and sucrose unit (glucose-fructose disaccharide) [1]. The location of linkage specified the type of fructan, in inuline the fructosyl residues are linked by β-2,1-linkages, while in levan (or Phlein), the fructosyl residues are linked by β-2,6-linkages with extensive branching through β- (2, 1) linkages [2]. Graminan is a third type of fructan with β-2, 1-linkages and β-2, 6-linkages [3]. Fructan is produced by some plants, fungi, yeast and bacteria; Plant fructan is synthesized from sucrose by recurrent fructosyl transfer from a fructosyl donor, with a terminal glucose unit, and in the present of sucrose-sucrose fructosyl transferase enzyme which catalyses the transfer of a fructose molecule from one sucrose molecule to another, and formation of kestose [4]. Plant fructan synthesized in the form of linear inuline consisting of B (2→1) fructofuranosyl units or more complex and branched levan consisting of B-(2→6) fructofuranosyl units [4]. Microbial fructan is synthesized by recurring transfer of fructose from a fructosyl donor to the growing fructan chain, with much higher degree of polymerization (up to 100,000) than plant fructan (up to 150) [5]. Fructan producing bacteria were reported from the following genera: *Pseudomonas*, *Zymomonas*, *Xanthomonas*, *Azotobacter*, *Erwinia*, *Streptococcus*, *Bacillus*, and *Arthobacter* [6, 7]. Fructan can be hydrolyzed by fructanases (inulinase and levanase) to generate fructose [8], mammals do not produce fructanases, and hence fructan is considered indigestible polysaccharides [6,9], therefore serve as substrate for utilization by the intestinal microflora and undergo fermentation in the colon by the colonic microflora resulting in the formation of acetate, lactate, and short chain of fatty acid which benefit the host health by simplifying the absorption of minerals and improving the host health [10].

Fructan and short-chain fructo oligosaccharides (FOS) are considered as representative prebiotics due to their ability to preferentially stimulate the growth of intestinal bifidobacteria [11].

Prebiotics is one of a non digestible food ingredient, which enhance the host health by stimulation the growth of colonic bacteria. The plentiful in the number of bifidobacteria and lactobacilli in the human and animal large intestine due to the ingestion of fructan present several benefits to their host, such as the competitive exclusion of intestinal pathogens, reduction of serum cholesterol, increasing

calcium and magnesium absorption, prevention of colon cancer, and production of B-vitamins [12].

Prebiotics also can be defined as enhancers of probiotic strains or beneficial endogenic strains of the gastrointestinal tract, therefore prebiotics selectively stimulate the growth of these microorganisms [13].

A probiotic is a mono - or mixed culture of living microorganism which beneficially affects the host by: Improving the properties of the indigenous population of gastro intestinal microorganisms, inhibition of pathogenic microorganisms, increasing the immune response and reduction of cholesterol levels with antimutagenic, anti carcinogenic activity [12, 14].

Bifidobacteria and lactobacilli have been used as probiotics, the beneficial effects of lactobacilli have been attributed to their ability to suppress the growth of pathogenic bacteria, possibly by secretion of antibacterial substances such as lactic acid, peroxide and bacteriocins [13,14]. Bifidobacteria and lactobacilli possess intracellular B (2-1)-d-fructan–fructan hydrolyase activity making the fructan molecules an efficient substrate, dietary fructan that reach the colon may thus select for an enhanced growth and activity of the indigenous bifidobacteria and lactobacilli population [13].

The present study was carried out to detect the effect of fructan that extracted from *Pseudomonas putida* to enhance and increase the viable cell number of *Lactobacillus acidophilus*.

Materials and Methods

Collection of samples

Ten food samples (rice, meat, cream, cheese, potato, bread, chicken, tomato, apple and peach) were collected from different local markets in Baghdad governorate in clean sterile plastic containers; each food sample was given a special code number, transported to the laboratory until using.

Isolation and identification of bacteria

Half-gram of each food sample was added to 4.5 ml of sterilized peptone water, mixed thoroughly and tenfold serial dilutions were done, MacConkey agar was prepared and inoculated with 100 µl from the appropriate dilution (1×10^7), incubated at 37°C for 24 hrs. Fluorescing colonies was taken and streaked again on MacConkey agar plate and these steps were repeated several times till a pure culture was obtained and for full identification of bacterial isolates, VITEK 2 compact were done [15, 16].

Production and Extraction of fructan from *Pseudomonas putida*

Brain Heart Infusion broth (BHI) was used to activate bacterial culture, the activated bacterial culture was streaked on YPS agar plates (1% yeast extract, 2% peptone and 5% sucrose), incubated at 30^oC for 48 hrs. Bacterial colonies with sticky appearance were selected for production and extraction of fructan [17, 18].

Identification of fructan by Fourier Transform Infrared Spectroscopy (FTIR)

For complete the identification of fructan, (FTIR) spectroscopy was done as follows**:** Potassium bromide was mixed with the dried samples in ratio of 1:10 (w/w), and compressed to form a thin tablet. The tablet was instantly analyzed with a spectrophotometer [19].

Study the effect of fructan to enhance the growth of *Lactobacillus acidophilus*

To study the effect of fructan as enhancer for the growth of *Lactobacillus acidophilus*, MRS growth medium (De Man, Rogosa and Sharpe agar) was supplemented with 1% of fructan powder and the bacterial number was estimated before and after supplemented of fructan to the culture medium as follows [20]:

1. *Lactobacillus acidophilus* was activated in MRS broth and incubated an aerobically at 37^0C for 24 hrs.
2. Control tube and test tube were prepared (control tube contained 10 ml of MRS broth+0.01 ml activated bacterial culture, while test tube contained 10 ml of MRS broth +1% fructan + 0.01 ml activated bacterial culture).
3. In order to calculate the initial cells number, tenfold serial dilution was done from (10^{-1} - 10^{-10}) for both tubes.

4. MRS agar plates were prepared and inoculated with 0.01 ml from each dilution from both tubes, incubated an aerobically at 37^0C for 48 hrs.
5. To calculate the increasing in bacterial cells number, both tubes were incubated an aerobically at 37^0C for 24 hrs, after that, serial dilutions were done from 10^{-1}-10^{-10} for both tubes.
6. From appropriate dilution (1×10^6) of both tubes, 0.01 ml were transferred and spread on MRS agar plates, incubated an aerobically at 37^0C for 48 hrs.
7. The viable cells number/ ml were calculated according to the formula:

CFU= number of colonies × dilution factor

8. Increasing in viable cells number were calculated according to the formula:

$$\text{Increasing in viable cells number (\%)} = \frac{\text{Viable cells number – Initial cells number}}{\text{Initial cells number}} \times 100$$

Results and Discussion

Isolation and Identification of *Pseudomonas putida*

Ten food samples were taken and collected in clean sterile plastic containers. Study the morphological characteristics and microscopic examination, revealed that twenty-five of bacterial isolates were belonged to *Pseudomonas* spp. [21, 22].

Pseudomonas spp. was characterized by fluorescing colonies on MacConkey agar; Microscopic examination proved that it was Gram-negative bacteria, flagellated and non-spore former and for full identification of *Pseudomonas putida* VITEK 2 compact were done as showed in Fig.(1).

bioMerieux Customer: **Microbiology Chart Report** Printed Jun 23, 2014 09:45 CDT

Patient Name: faeza 1 Patient ID: 130-6-1
Location: Physician:
Lab ID: 130-6 Isolate Number: 1

Selected Organism : Pseudomonas putida

Source: 9-11 Collected:

Comments:	

Identification Information	Analysis Time:	8.00 hours	Status:	Final
Selected Organism	91% Probability Bionumber:	Pseudomonas putida 1003013101500352		
Organism Quantity:				
ID Analysis Messages				

Biochemical Details

2	APPA	+	3	ADO	-	4	PyrA	-	5	IARL	-	7	dCEL	-	9	BGAL	-
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	+	15	OFF	-
17	BGLU	-	18	dMAL	-	19	dMAN	-	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	+	26	LIP	+	27	PLE	-	29	TyrA	+	31	URE	-	32	dSOR	-
33	SAC	-	34	dTAG	-	35	dTRE	-	36	CIT	+	37	MNT	-	39	5KG	-
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	-	45	PHOS	-
46	GlyA	-	47	ODC	-	48	LDC	-	53	IHISa	+	56	CMT	+	57	BGUR	-
58	O129R	+	59	GGAA	-	61	IMLTa	+	62	ELLM	-	64	ILATa	+			

Fig.(1): VITEK2 compact identification for Pseudomonas putida.

Production and Extraction of Fructan from *Pseudomonas putida*

Fructan was extracted from the production medium (YPS broth) with three volumes of 95% cold ethanol; the amount of fructan was established by measuring the final dry weight of the precipitate after centrifugation. Fructan appeared as white powder and the dry weight was 3.6mg/100 ml.

Cold ethanol lowering polysaccharide solubility, thus it was permitting their separations from the production media [23].

For the purification of fructan, repeating precipitation in ethanol and dissolution in water was performed [24, 25].

Identification of Fructan by Fourier Transform Infrared Spectroscopy (FTIR)

The infrared spectrum of this biopolymer showed the functional groups found in polysaccharides, which it was (C-O, C-H, O-H and C=O); C-H and O-H group bending in (1223.85cm^{-1} and 1450.03 cm^{-1}), C-H stretching in (2819.73 cm^{-1} and 3000.01 cm^{-1}), O-H stretching in (3390.63cm^{-1}and 3461.99 cm^{-1}), C=O and C-O stretching group in (1649.02 cm^{-1} and 1114.78 cm^{-1}) respectively. Fig. (2)

The absence of active groups present in nucleic acids and proteins demonstrates that the main component of the chemical composition of fructan compound is carbohydrates [26, 27].

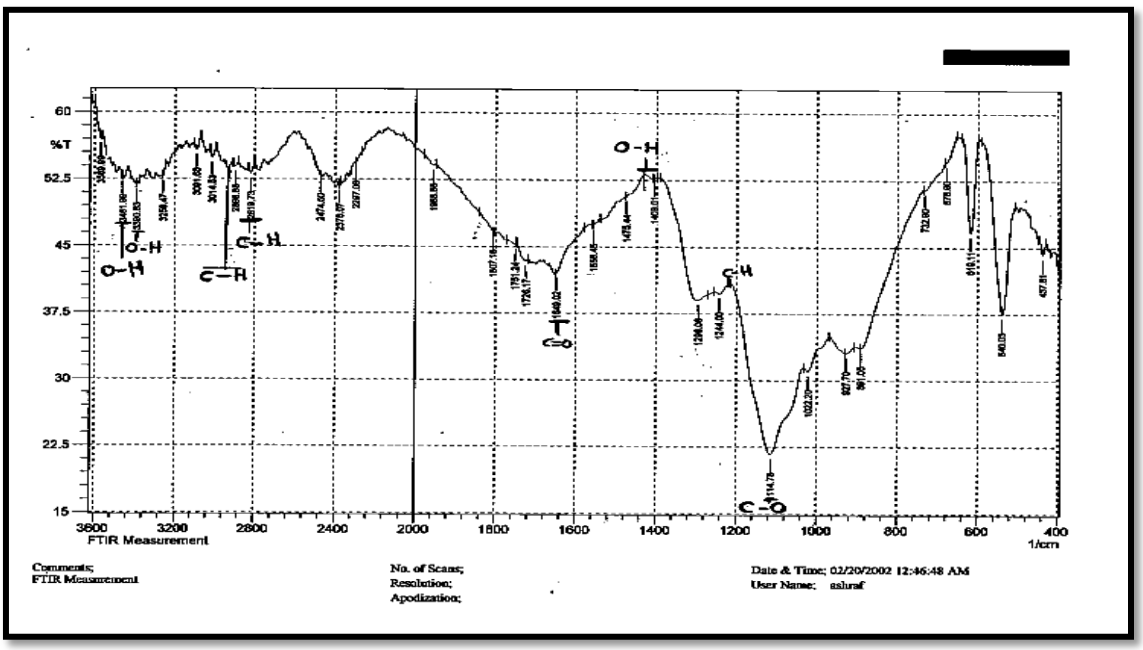

***Fig.(2)*: *FTIR analysis of fructan produced from Pseudomonas putida*.**

Study the effect of fructan as enhancer for the growth of *Lactobacillus acidophilus*

MRS medium was inoculated with *Lactobacillus acidophilus* before and after supplement the growth medium with 1% of fructan powder. The results was recorded depending on the viable cell number of bacteria on MRS and compared between the viable cell numbers of *Lactobacillus acidophilus* on MRS medium with and without supplement of fructan.

The results exhibited rising in the viable cells number of *Lactobacillus acidophilus* on MRS that supplied with fructan compared with control as showed in Table (1).

Table (1)
Effect of fructan as enhancer for the growth of Lactobacillus acidophilus.

MRS medium	*Initial cell no. (Log CFU/ ml)*	*Viable cell no. after 48hrs. (Log CFU/ml)*	*Increasing in viable cell no. (%)(a)**	*Increasing in viable cell no. (%) (b)**
Control	4.07	6.57	61.42	31.5
1% fructan	4.17	6.84	64.02	46.5

**** (a) The percentage was calculated according to log of viable cell number.***
**** (b) The percentage was calculated according to cell number.***

From the table; it was clear that the existence of fructan in MRS medium had a role as enhancer for the growth of *Lactobacillus acidophilus* in addition to increase the viable cell number of it.

The logarithmic increasing in the viable cell number of *Lactobacillus* after the addition of levan at different concentration to growth medium proved that levan enhanced the growth of *Lactobacillus acidophilus* [20].

Lactobacillus acidophilus used specific enzymes (fructosidases) to hydrolyse B-(2→6) linkages present in carbohydrates structures and utilize it as substrate to enhance the growth of it [28].

The lowering in intestinal pH, resulting from the fermentation of fructo oligosaccharide (FOS) by the mean of colonic normal flora have imprint in reducing the pathogenic bacteria by making the pH of the intestinal unsuitable for the growth of it [29].

Conclusion

Fructan extracted from Pseudomonas putida showed the ability to increase and enhanced the growth of Lactobacillus acidophilus when supplement the growth medium of Lactobacillus acidophilus (MRS) with 1% of fructan powder, FTIR analysis exhibited the essential groups present in fructan structure which it was C-O, C-H, O-H and C=O.

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