Production, Analysis and Optimization of Inulin Produced from *Pseudomonas fluorescens*

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Abstract

Inulin is a natural polysaccharide produced from organisms and microorganism. Inulin is a type of fructan that α-amylase cannot digest it and cannot hydrolysis by hydrolytic enzymes. It was used to get non-fat fermented milk and it's a type of prebiotics that induce growth and activity of probiotics bacteria, thus improving the health. *Pseudomonas fluorescens* have the capacity to produce inulin when grown in media supplied with sucrose. Twenty-two of bacterial isolates were belong to *Pseudomonas fluorescens* depending on structural features, microscopic checking, biochemical analysis and flourescent pigments that produced on King B medium. Ten isolates of *P. fluorescens* had strongly degree of mucous growth on solid production medium, the gummy and mucous manifestation on agar medium containing sucrose were give rise to inulin production. Inulin produced from *P. fluorescens* was analyzed by Fourier Transform Infrared Spectroscopy to detect functional groups which it was (C-O, CH, OH and C=O) and by Thin Layer Chromatography to determine its components of monosaccharide. Inulin distinguished as dark spot on white background and the Rf of it was (0.58). The best product of inulin were in production salt agar medium containing 20% sucrose, pH =7, Temperature =37^{0}C without nitrogen sources and the inoculum size 1%, it was (3.2 gm⁄100 ml). The effects of bacterial inulin on the growth of *Saccharomyces cerevisiae* was studied by culturing it in medium supplemented with 3% of bacterial inulin at 30°C for 48 hrs. The results showed there was no remarkable effect of inulin on *Saccharomyces cerevisiae* growth in comparison to control.

Keywords: *Inulin, Fructan, Levan, Pseudomonas fluorescens, Saccharomyces cerevisiae, Probiotic, Prebiotics, Biochemical test.*

Introduction

Inulin is a natural polysaccharide produced from organisms and microorganism [1, 2]. It is a type of fructan that α-amylase cannot digest it and cannot hydrolysis by hydrolytic enzymes [3,4]. Inulin have protective effect on the survival and activity of lactic acid bacteria when storage and use it as final product [5,6], and it was stimulated the growth of probiotic bacteria thus improving the health [7].In food technology inulin use to improve body mouthful, as stabilizers, fat replacers, and flavour enhacers [8,9]**.** *Pseudomonas fluorescens* capable for adaptation at different environments by extracellular substances [10]. Living micro-organisms and its products are widely used for therapeutic purposes, *Saccharomyces cerevisiae* also possess some medicinal effciency, and the benefcial properties of it are well documented [11,12].

At present day many pharmaceutical preparations with microorganisms products are commercially available [13, 14].

The aim of this study was to seek the capacity of local isolate of *Pseudomonas fluorescens* to produce inulin in different conditions and the effect of inulin on *Saccharomyces cerevisiae* growth.

Materials and Method

Samples collection

Thirty-five of different food samples were collected from different local markets in Baghdad governorate in sterilized utensil and imparted to the laboratory until using.

Sorting and Identity of bacteria

Half-gram were taken from specimen and 4.5 ml of sterilized peptone water were added, next dilutions were done, MacConkey agar was prepared and inoculated with 100 µl from the adequate dilution (1×10^7), incubated at 37°C for 24 hrs. Fluorescing colonies were taken

and streaked again on the same agar medium several times till a pure culture was obtained. Bacterial isolates were identified by using selective medium (King B medium), structural features, microscopic checking and biochemical analysis [15].

Checking of Inulin generating isolates

Purified bacterial isolates were activated in Brain Heart Infusion broth (BHI), after incubation periods (0.1 ml) of culture suspension was streaking on production medium (3gm KH2PO4, 3gm K2HPO4, 0.5 gm MgSO4.7H2O, 20% w/v of sucrose and 2% agar- agar), incubated at 37^0C for 24hrs. Mucoid consistence of bacterial colonies gave marked of inulin production.

Quantitative checking in liquid medium

The highly mucous isolates were selected, 10 ml of (BHI) broth were prepared and inoculated with bacterial isolates then incubated for 18hrs at 37^0C, after incubation periods 100 ml of mineral broth (supplied with 20% of sucrose) was cultivated with 1ml of isolates, incubated for 24hrs, 37^0C. Centrifuge were using (6000rpm, 30 minutes) for extracting inulin by mixing the cell free supernatant with ethanol at rate (1:4) and allowed to stand overnight, the aqueous layer was removed and the layer of inulin was collected in sterilized petri-dish and dried at 60^0C.**[16].**

Diagnosis of Inulin:

a) **Fourier Transform Infrared Spectroscopy (FTIR):** Inulin dried weight was analyzed by using the crystal of potassium bromide (KBr) at rate 1:10 (w/w) [17]

b) **Thin Layer Chromatography (TLC):**This method was done according to (Shida *et al*.(2002) [18] as following:

1. Inulin was dissolved (0.01gm) in 1N HCL and incubated at 70^0C for 3 hrs.

2. About 10 µl of this suspension was taken and spotted plentiful of time away from the below end of TLC plate.

3. Sucrose, Fructose and Glucose solutions were destined and spotted in the same manner and they used as marker.

4. The plate of TLC placed in a closed jar containing separation system (butanol: propanol: D.W.: acetic acid at proportion of (7:5:4:2,v:v:v:v), until spread through the plate at 15 cm.

5. TLC taken and drying up.

6. Dried TLC spirt with TLC diagnosis solution (ethanol:H_2SO_4 at rate 9:1, v:v.), put at (90^0C, 10 minutes) [19].

7. Inulin demonstration as dark spot and the space of it were determination.

8. Relative flow (Rf) estimation as the following:

Distance of the sample mobilized across the plate / Distance of the solvent [20].

Influence of some factors on inulin product

1. **Carbon**

A. Production broth (supplied with 20% of glucose and lactose) were prepared**,** inoculated with bacterial growth culture**,** incubated for 24hrs, 37^0C.

B. Extraction of inulin and determination of dry weight.

C. Comparison with inulin dry weight which extracted from mineral broth containing 20% sucrose.

2. **Nitrogen**

A. Production broth with best carbon source were prepared with addition of 1% (yeast extract and peptone), inoculated with bacterial growth culture**,** incubated for 24hrs, 37^0C.

B. Extraction the product and determination the weight.

3. **pHs**

A. Production broth with best carbon source prepared at pHs (5, 6, 7, 8, 9 and 10), inoculated with bacterial growth culture**,** incubated for 24hrs, 37^0C.

B. Extraction the product and determination the weight.

4. **Temperature**

A. Production broth with best (carbon source and pH) prepared**,** inoculated with bacterial growth culture**,** incubated for 24hrs, at (37, 45 and 50^0C).

B. Extraction the product and determination the

weight.

The effect of inulin on *Saccharomyces cerevisiae* growth

In order to investigate *Saccharomyces cerevisiae* growth in existing inulin, *S.cerevisiae* was cultivated in potato dextrose broth, incubated at 30°C for 48hrs then centrifugation for 15 min with 2500×g at 4ºC. The precipitate was taken and washed by (PBS) (0.1 M phosphate buffer pH 7.4, 0.9% saline) and re suspended in PBS.

The suspension was cultured in medium supplied with 3% inulin, incubated at 30°C for 48hrs. The turbidity of cultured medium was measured at 600 nm for up to 48 hrs and compared with control cultured medium free from inulin [21].

Results and Discussion

Sorting and Identity of *Pseudomonas*:

Thirty-five of different food samples were collected from different local markets in Baghdad governorate. Twenty-tow of this isolates were diagnosed as *Pseudomonas* according to structural features and microscopic checking [17]. Flourescent producing on King B medium and Microscopic examination showed Gram-negative bacilli, non spore former bacteria and biochemical analysis showed (urease+, oxidase +, Gelatine hydrolysis +, Catalase +, Starch hydrolysis -) these results showed that they were identified as strains of *Pseudomonas fluorescens* [22].

Checking of Inulin generating isolates

Pseudomonas fluorescens streaking on production medium; for checking their capability to generate mucus manifestation as marking for Inulin production.

Inulin production was varied from species to species. The gummy and mucous manifestation is coming from manufacturing of polysaccharide [23].

Quantitative checking in liquid medium

Ten isolates of *P. fluorescens* with strongly degree of mucous growth had selected for checking their capability to generate inulin in broth medium that supplied with 20% of sucrose.

The highly mucous appearance of these isolates were taken for creation of inulin in mineral salt broth and the highly product was 3.2 gm/100 ml.

Diagnosis of Inulin:

a) Fourier Transform Infrared Spectroscopy (FTIR): Inulin generated by *P. fluorescens* was analyzed by FTIR spectroscopy to detect the effective structure of inulin.

The results showed the presence of C-O stretching group in 1122.49 cm^{-1} , bending group CH,OH in 1336.58 cm^{-1} and 1434.23 cm^{-1}, stretching C=O in1649.02cm^{-1}, stretching CH in 2891.10 cm^{-1} and 2931.60 cm^{-1}, stretching OH in 3367.48 cm^{-1} and 3431.13 cm^{-1}as showed in figure (1). All these groups (C-O, CH, OH and C=O) are functional groups found in carbohydrates [17].

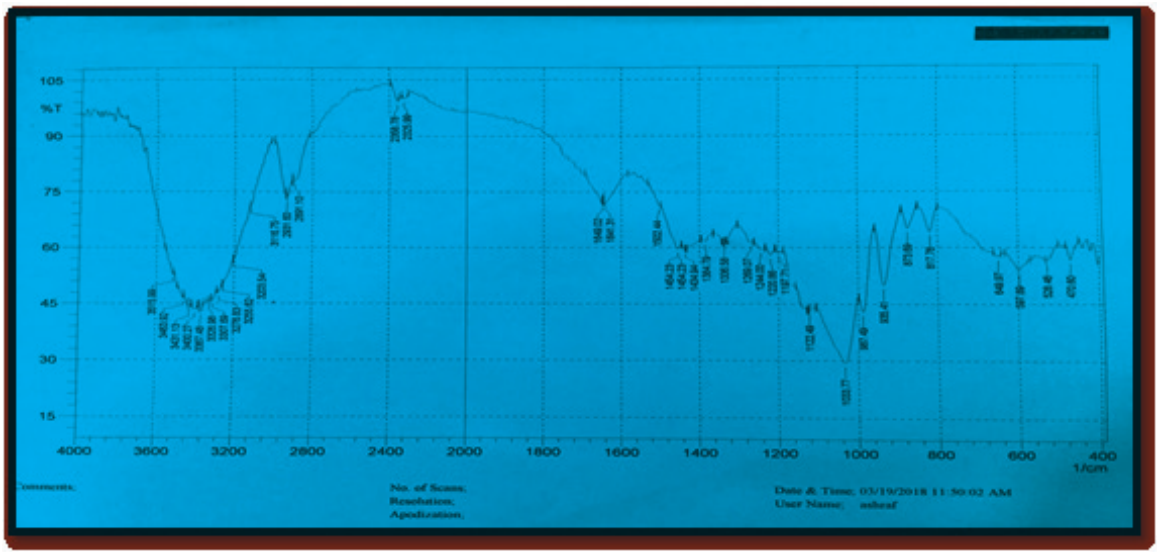

Figure1: FT-IR for inulin production from *P. fluorescens*

Kazim AR. (2015) [17] reported that polysaccharide extracted from *Pseudomonas* does not contain lipids or nucleic acid in structures.

b) Thin Layer Chromatography (TLC): Inulin production from *P. fluorescens* was analyzed by TLC chromatography to determine its components of monosaccharide.

Inulin extracted from *P. fluorescens* was hydrolyzed with HCL before application on TLC and standard sugars (glucose, fructose and sucrose) were prepared and used as marker.

These sugars distinguished as dark spot on TLC plate when using TLC diagnosis solution (ethanol:H_2SO_4 at proportion of 9:1, v:v.) as showed in figure (2).

Rf of these sugars (inulin, fructose, sucrose and glucose) were estimated according to Ghosh, S. and Chandra, A. (1980) [20] and the results were (0.58, 0.59, 0.57 and 0.55) respectively.

Rf for the same compound differed according to (separation system, diagnosis solution and type of solvent) [24].

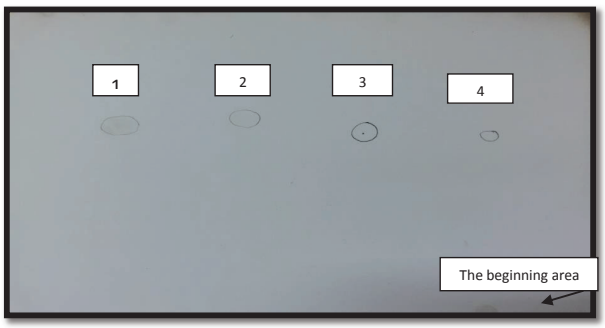

Figure 2: TLC for Inulin production from *P. fluorescens*: Note: (1) inulin, (2)fructose, (3)sucrose and (4)glucose.

Influence of some factors on inulin product

1. **Influence of carbon**

Inulin product may be Influence by the (type, kind and concentration) of carbon substance.

For investigation the influence of carbon, production medium supplied with 20% of glucose, lactose and sucrose inoculated with *Pseudomonas flourescens* and incubated for 24hrs, 37^{0}C.

From study of the results, the best product of inulin from *Pseudomonas flourescens* was obtained from production medium supplied with sucrose (3.2 gm/100 ml) and the minimum productivity was in existence of glucose (0.2gm/100 ml) as shown in figure**(3).**

The amount of inulin was varied and the highest inulin production was produced when *Pseudomonas flourescens* was cultivated in medium containing 20% of sucrose.

Microorganisms catabolized polysaccharide such as sucrose by levansucrase enzyme which degradation of sucrose [15].

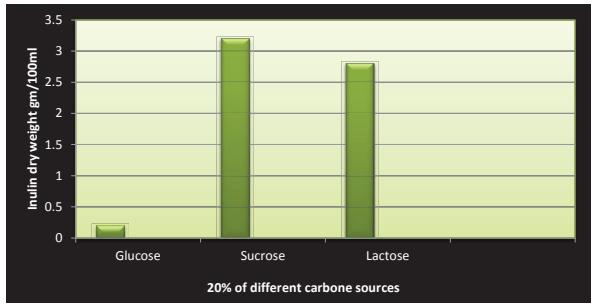

Figure 3: Influence of carbon sources on the productivity of inulin from *Pseudomonas flourescens*

2. **Influence of nitrogen**

By studying the results showed that there was reducing in productivity of inulin when supplying production medium with 1% of yeast extract and peptone.

The productivity decrease from (3.2 gm/100ml) to (2.1 gm/100) when supplied production medium with yeast extract and to (1.8 gm/100ml) in the present of peptone in medium as shown in figure (5).

Microorganisms required nitrogen to complete the metabolic pathway, also nitrogen enhances and increase the microorganism growth; these increasing in growth may be decreased the production of inulin.

The effect of yeast extract in production medium was studied previously, rising concentration of yeast extract responsible for an increase in the *Zymomonas mobilis* and reduction the productivity of inulin, yeast extract also improves and enhancement of substrate consumption, increasing in consumption of substrate verified only a small part of consumed sugar and converted it to Inulin [25].

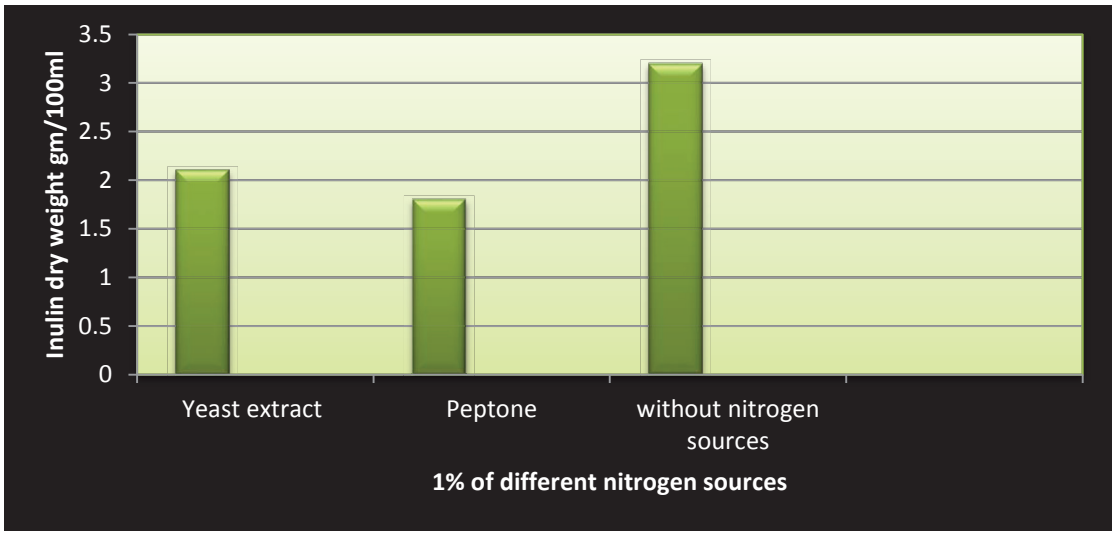

Figure 4: Influence of carbon sources on the productivity of inulin from *Pseudomonas flourescens*

3. Influence of pHs

From the results revealed; inulin productivity was varied with pH of production medium, the productivity decrease in pHs less than 7 and more than 7 as showed in figure (5).

Inulin is chemically stable in a natural and alkaline environment and its stability decreases in an acidic environment [25].

4. Influence of temperature

It was revealed that the best productivity of inulin at 37°C (3.2 gm/100ml) and the productivity was decrease at temperature above than 37°C, it was 2.1gm/100ml at 45°C and 0.1gm/100ml, figure (6).

From the result, the best temperature for the productivity was 37°C and at this temperature enzyme responsible for inulin may be synthesis.

Fructosyltransferase enzymes (FTFs) is bacterial enzymes responsible for synthesis of inulin and frctun from sucrose in three reactions [25].

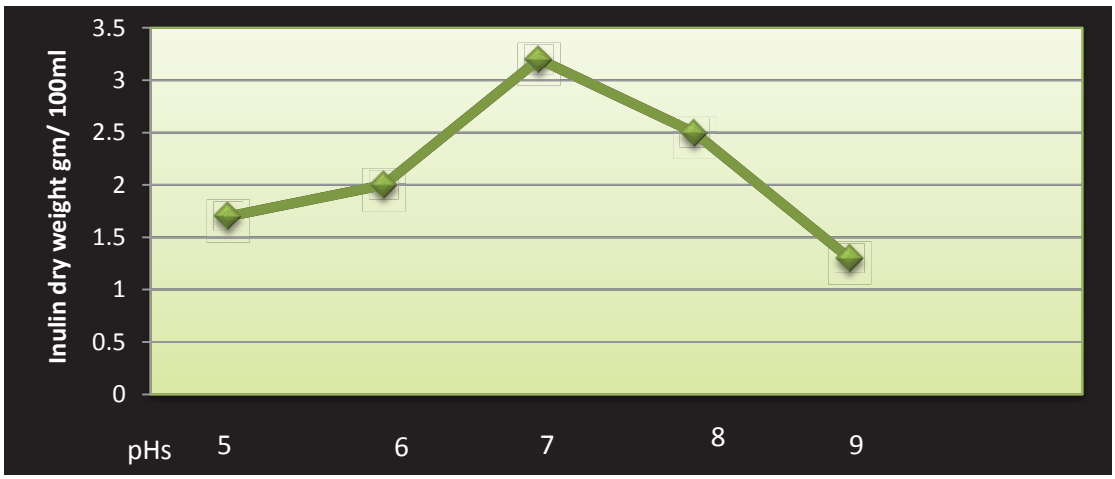

Figure 5: Influence of pHs on the productivity of inulin from *Pseudomonas flourescens*

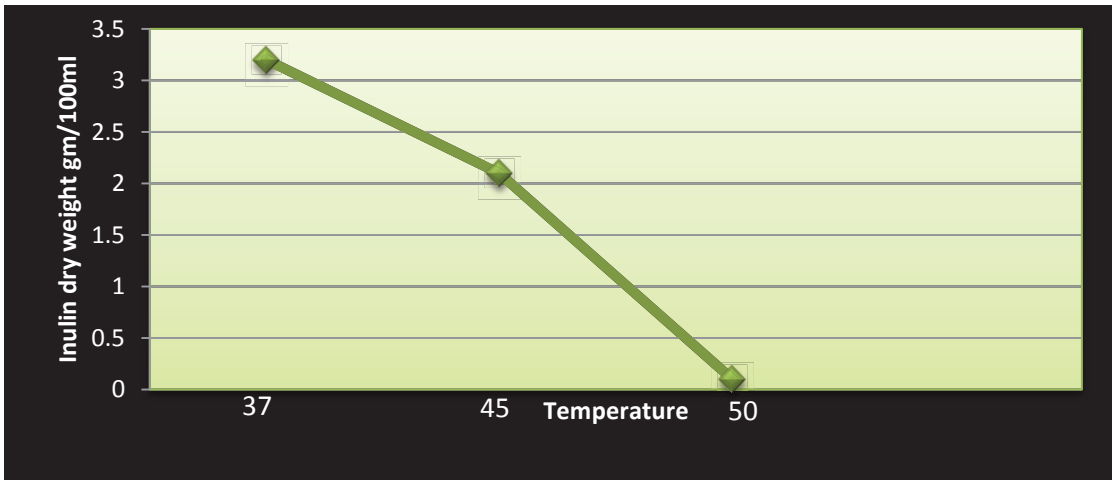

Figure 6: Influence of temperature on the productivity of inulin from *Pseudomonas flourescens*

Fungal Growth study

In order to investigate *Saccharomyces cerevisiae* growth in existing inulin, *S.cerevisiae* suspension was cultured in medium supplied with 3% inulin, incubated at 30°C for 48hrs. The turbidity of cultured medium was measured at 600 nm for up to 48 hrs and compared with control cultured medium free from inulin.

From the results of OD,(OD of control (2.8278), OD of test medium supplied with 3% inulin was 3.0158) there was no remarkable effect of inulin on *S.cerevisiae* growth when compared with the growth of control.

S.cerevisiae had not enzymes responsible for degradation of inulin and used it as carbon sources.

Inulinases hydrolysis of inulin to glucose and fructose; already these molecules can be used as carbon sources to produce many beneficial products for microorganisms.

Conclusion

These studied seeking for the capacity of inulin production from local isolate of *Pseudomonas fluorescens*. The results showed no remarkable effectiveness of inulin on the proliferation of *Saccharomyces cerevisiae* compared with control.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: The authors declare that they have no conflict of interest.

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