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Research Article

Use of date syrup as alternative carbon source for microbial cultivation

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In the present work, date syrup and date fruit soaked water as alternative carbon source for biomass production of *Bacillus megaterium* as model organism was optimized. Maximum biomass production was obtained on 2.8, 4.1 g/l for molasses and date fruits soaked respectively. This source was substantially greater than could be attained on media that used various other carbon sources. The optimal medium for producing the biomass was a mineral medium formulated with 8% of date syrup as the carbon source and 0.5 g/L $(NH_4)_2SO_4$as the nitrogen source. At optimal fermentation time of 48 hrs, at 30°C.Water soaked and date syrup can be used to inexpensively produce biomassin batch fermentations using *B. megaterium* as phosphorus solubilizing soil bacteria. Farther more studies should be focused on agriculture cheapest sources as nature alternatives for carbon ear nitrogen sources.

Keywords: Date syrup, microbial cultivation, fermentation optimization, biomass, *Bacillus megaterium.*

INTRODUCTION

Bacillus megaterium is an aerobic gram positive, endospore forming, rod shaped bacteria. It is considered aerobic. It is found in soil and considered a saprophyte. *Bacillus megaterium* is latin for the big beast because it is an extremely large bacteria, it is about 100 times as large as *E. coli*. Due to its immense size, about 60 micrometers cubed, *B. megaterium* has been used to study structure, protein localization and membranes of bacteria since the 1950's. Most notably, *B. megaterium* is the organism that was used by Lwoff and Guttman in the studies that discovered lysogeny, it is both a desirable cloning host and produces a large variation of enzymes (*Bergey's 1994; Al Eid 2006; Al-Fayiz* et al., 2007).

Chemical fertilizer application is an effective method to increase yields, but is costly and may also lead to environmental problems. In particular, phosphorus fertilizers present a serious risk of cadmium accumulation in soil (*Al-Fayiz* et al., 2007).

The bacteria used as phosphorus bio fertilizers could contribute to increasing the availability of phosphates immobilized in soil and could enhance plant growth by increasing the efficiency of other nutrient. *Bacillus megaterium* var. *phosphaticum*, a phosphorus solubilizing bacteria, releases acid into the rhizosphere to enhance nutrient uptake (*Al-Farsi* et al.,2007; *Vazquez et al., 2003).*

Increasing world population and the resultant food crises has shifted emphasis to the availability of 'waste' products of agriculture that could be utilized for all food shortage. In many of the developing countries where major nutritional problems exist, excess of materials rich in carbohydrates are produced.

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These materials can be utilized in fermentation processes to produce microbial protein which in turn can be used to upgrade both human food and animal feeds. Traditional protein sources are relatively more expensive and, the absence of well-developed technology facilities have contributed to losses incurred through spoilage of even the limited available sources. Consequently, there is need to explore alternative ways of meeting the protein demands. Compared to the developed countries (mainly the U.S.A, U.K and Japan).

Microorganisms need nutrients, a source of energy and certain environmental conditions in order to grow and reproduce. In the environment, microbes adapt to the habitats most suitable for their needs while in the laboratory, these requirements must be met by a culture medium. So growth media are used for various purposes including the identification of unknown microorganisms, as well as the production of large quantities of microbial populations for commercial uses as in biotechnology. Numerous types of media are available commercially including some that may have added compounds that either enhance growth or suppress outgrowth of competing organisms. Complex media are rich in nutrients; they contain water soluble extracts of plant or animal tissue. Usually, sugar or glucose is added to serve as the main carbon and energy source. The combination of extracts and sugar creates a medium which is rich in minerals and organic nutrients, but since the exact composition is unknown the medium is called complex. Selection of preferred media is based on how it affects the microorganism's growth and other physiological functions and the purpose of research.

Date syrup is a potential substrate that has been utilized for ethanol, citric acid and amylase production by some fungal strains (Acourene and Ammouche, 2012 the use of date syrup for producing pectinases for possible use in cotton scouring. Date (Phoenix dactylifera L.) is an important crop in desert regions of the Middle East and contributes significantly to human nutrition in some regions. Date fruit is highly nutritious and rich in calories (Al Hooti et al., 2002). Date fruit is boiled and then dried for storage. This process results in rich waste syrup that is potentially useful as a fermentation substrate. Large quantities of waste date syrup are produced, for example, in Sindh region of Pakistan. Pakistan also has a very substantial cotton textile industry. Use of the waste syrup to produce pectinases can potentially eliminate a pollution problem, improve revenues in date processing and reduce the cost of importing expensive pectinases for use in production of cotton textiles. (Al Hooti et al., 2002)

Taniwaki et al. (2002), reported studies comparing culture media simulate and Petri film for enumeration of yeasts and molds in food. The efficacy of three culture media; dichloran rose Bengal chloramphenicol (DRBC), dichloran 18% glycerol agar (DGA) and potato dextrose agar (PDA) supplemented with two antibiotics, were compared with the simulate and Petri film techniques for mold and yeast enumeration by these scientists. Then, the following foods were analyzed: corn meal, wheat flour, cassava flour, bread crumbs, whole meal, sliced bread, ground peanuts, mozzarella cheese, grated parmesan cheese, cheese rolls, orange juice, pineapple pulp, pineapple cake and mushroom in conserve.

Qiyun and Liang, (2004) studied the use of potato processing waste as a fermentation substrate for the production of single cell proteins (SCP) for use in supplementation of animal feeds (Qiyun and Liang, 2004). Comparisons were conducted using raw and steamed potato waste; both fermented using a single microbial strain and also the solid-state fermentation of wastes with a mixed microbial culture. Composition before and after fermentation was determined and this showed that the crude protein contents were 13.4, 18.53 and 22.16%, for the raw, steamed and solid-state treatments, respectively.

The conventional medium palm kernel agar (PKA) for the recovery of aflatoxigenic fungi from mixed cultures and the detection of aflatoxigenic fungi and direct visual determination of aflatoxins in agricultural commodities was assessed by Atanda et al., (2006). The medium was able to efficiently detect aflatoxin production through direct visual observation of fluorescence. It can be routinely used as an alternative culture medium for screening aflatoxigenic fungi and direct visual determination of aflatoxins in agricultural commodities since it is faster and has a unique pink background for easy identification (Atanda et al., 2006).

Various factors are known to influence fermentation processes. These include carbon and energy source requirements, oxygen demand and supply, temperature, pH, Nitrogen, Phosphorus and potassium.

The major carbon and nitrogen source of fermentation media are soybean meal, molasses, corn steep liquor, sulphite waste liquor, cotton seed meal, yeast extract, peptone etc. Calcium chloride, ammonium phosphate and potassium phosphate are incorporated for enhanced growth. Microbes grow more vigorously on complex media than in mineral media, because the former contain biosynthetic precursors that can be channeled directly into anabolic pathways reducing the need to produce them and saving metabolic energy. Pharmamedia, molasses, corn steep liquor, sulphite waste liquor are used as fermentation substrates for microbes.

Date fruit (*Phoenix dactylifera*) is an important product in many Gulf countries. Contains carbohydrate (total sugars, 44–88%), fat (0.2–0.5%), protein (2.3–5.6%,(dietary fiber (6.4–11.5%), minerals (0.1 to 916 mg/100 g date), and vitamins such as vitamin C, B_1, B_2, A,

riboflavin, and niacin. Date is becoming an important commercial crop in the producing countries which have adopted advanced biotechnological approaches to increase yield significantly. Date processing industries, however, have not expanded at the same rate. Furthermore, due to persistent rain and stormy conditions, a large amount of the harvested dates become dusty and are damaged by birds and insects (Murugalakshmi and Sudha (2010).).

In this study some factors affecting the yield of cell biomass production, natural alternative of carbon source by *Bacillus megaterium* are ascertained.

In this study, one date variety (Khalas) has been selected due to its availability in large quantities, nutrition compound and its low cost. Dates are a high energy food containing most of the basic dietary elements such as sugars, proteins, fats, and minerals and are also important raw materials for many food products. However, it is important to use the best techniques for date syrup extraction to maximize fermentable sugars extraction. Three extraction techniques can be used for date syrup preparation. These techniques are classical extraction, fluidized bed, and super critical fluid extraction. The classical technique is the simplest and cheapest; however, different factors such as temperature and water rate can influence the extraction technique.

The fleshy part of dates contains carbohydrates, vitamins, salts and minerals, protein and small amounts of fats, oils, and acids. Dates are composed of large amounts of reducing sugars including sucrose, glucose, and fructose

MATERIALS AND METHOD

Isolation and identification of strain

Rhizosphere soil samples were collected from Qassim area Saudi Arabia. The Promising Research Center in Biological Control and Agricultural Information. Qassim University. P.O. Box 6622, Burydah 51452. Kingdom of Saudi Arabia

Serially diluted with sterile distilled water up to10^{-7}, where the dilution was started from 10^{-2}(1g of sample in 100ml-distilled water). From the above dilutions 10^{-2}, 10^{-3} and 10^{-4}were adopted for pour plate technique and the plates were incubated at 37°C for 24hrs. The colonies were confirmed by biochemical method (*Bhutto M. A. and D. M. Umar* (2010).), (*Bergey's,1994).*

Preparation of seed culture: 5 ml of sterile distilled water were added to bacterial slants (grown for 24 hrs at 30°C) and shacked well for 1m. This was then transferred to 50 ml of Schlegel's mineral medium in250ml capacity Erlenmeyer flasks and incubated at 30°C on an orbital shaker at 140 rpm for 24 hrs.

5 ml of seed culture were transferred to 100 ml of MSM medium in 250 ml capacity Erlenmeyer flasks and shacked at 140 rpm at 30°C for 24 and 48 hrs. (*Bergey's, 1994).*

Preparation of date syrup (Dips): The date cultivar (variety) **(Khalas**) used in this experiment, were purchased from the local market in Riyadh, its economic affordability and its availability in large quantities in Saudi Arabia. Khalas was washed, removed stones and grounded. Two and half liters of hot water at 80-85°Cwere added to 1 kg of date, homogenized and filtered. The syrup obtained was centrifuged at 15000 rpm for 10 minutes to separate the cellulose debris. The collected supernatant was used as culture medium.The syrup is sterilized during 20 minutes at 120°C.

Preparation of date water soaked: The extraction of date syrup was performed as described by Al-Eid (2006) with some modification. The dates were sliced to pieces. Fifty grams of stone-free dates were soaked in 225 ml distilled water for 10 min.The soaked dates were then aseptically blended in a sterile blender for 5 min at low speed. The homogenized mixture was transferred to a 500 ml Erlenmeyer conical flask and placed on magnetic stirrer for 30 min at 30°C. The slurry was filtered through a cloth using hand press. The collected raw date juice was then centrifuged at 7,000 x g for 30 min. The produced date syrup was autoclaved and then packed in sealed glass bottles and stored at room temperature.

Effect of different concentrations of date syrup

The biomass production was evaluated in 50 batch fermentations at 30°C with 1 L working volume for 30hrsincubationper batch. The repeated batch fermentations of MSM media were investigated with different concentrations of the date syrup including 2.0, 4.0, 6.0, 8.0, and 10.0 % (v/v), and 10 gm./glucose was used as control. The nitrogen source was 0.5 g/l of$(NH_4)_2SO_4$. All patches were tested during different incubation time (*Al Eid (2006)*).

Effect of different nitrogen sources

The accumulation of the biomass with different nitrogen sources and different concentrations of date syrup was investigated. Fifty repeated batch fermentations at 30°C with1L working volume and 30 hrs incubations per batch were run. The nitrogen sources (0.5 g/l) included $(NH_4)_2SO_4$, NH_4Cl, $NH_4H_3PO_4$, and CH_3COONH_4. The date syrup concentrations were 2.0, 4.0, 6.0, 8.0, and 10.0 % (v/v).

All data were analyzed at an alpha level of 0.05 or 0.10 using design by completely randomized design model using the analysis of variance module (3 R) all treatment means were separated using Fisher's protected least significant difference (LSD) mean separation.

Table 1. Optimization of *Bacillus megaterium* (log $_{10}$) using date palm syrup " dips" and soaked as alternative carbon source. N=0.35 g/l, pH=6, rpm=175 at 30^{o}C.

%	Water soaked dates	Molasses	MSM
2	3.1	2.1	5.0
4	3.5	2.5	5.1
6	4.0	2.7	5.2
8	4.1	2.8	5.5
10	3.7	2.8	5.5

Table 2. Effect of date syrup on utilization of total biomass of *Bacillus megaterium*. N=0.35 g/l, pH=6, rpm=175 at 30^{o}C.

	Biomass g/l										
%	1	2	3	4	5	6	7	8	9	10	control
Date syrup (dips)	2.5	2.5	2.7	3.0	3.7	4.2	4.3	4.6	4.5	4.5	
Water soaked dates	3.1	4.2	4.5	5.2	5.7	5.7	5.9	6.0	6.1	6.1	5.2

Table 3. Effect of different concentrations of nitrogen source onbiomassproduction by *Bacillusmegaterium* using 8 % date syrup as a carbon source when initial pH was 7.0 and incubated at 37°C for 7 hours.

N- Source	$(NH_4)_2SO_4$	NH_4Cl	$NH_4H_3PO_4$	CH_3COONH_4
Water Soaked 8%,Biomass g/l	6.2	5.2	5.5	5.7
Molasses 8%, Biomass g/l	5.3	5.2	5.1	4.8

RESULTS AND DISCUSSION

Batch fermentation for different concentrations of date syrup was used to stimulate the biomass production of *B. megaterium.*

However, the low and high concentrations of dates were not stable for biomass production (Table 1). General, biomass accumulation was significantly higher with date syrup at 8% .The biomass was 4.6 g/l and 6.0g/l for syrup and soaked dates respectively. The biomass was calculated based on the cellular dry matter.

The low concentration might have a low sugar content which was not a sufficient amount for biomass production.While, the high concentration might have contained some inhibitors or undesirable compounds such as acetic acid, propionic acid, butyric acid and formic acid (*Al Eid (2006)*,). which might have affected biomass production. These inhibitors can affect the microorganism's fermenting ability both by producing ethanol and by stopping growth *(Bhutto M. A. and D. M. Umar*(2010)), (*Khiyami M, Pometto AL, Brown R (2005).*).

The effect of alternative carbon sources on biomass production was tested by replacing glucose with the date palm syrup. The results are shown in tables (1and2). Compared to the other carbon sources, complex media based on water soaked and date syrup gave the highest biomass at 48 hrs of fermentation. The date syrup is a liquid which is produced as a by-product of date industry and contains (75% carbohydrates w/w) small amount of fats and proteins along with micro and macro elements (*Al-Farsi* et al., (2007); *Al-Hooti* et al., 2002) The date syrup based medium was the most effective probably because dates contain a significant quantity of biomass and this may induced the production of microbial enzyme's and biomass.

In view of its effectiveness and low cost, date syrup was used as the carbon source in all subsequent work. Further tests were done to elucidate the effect of the initial concentration of date syrup in the medium on production of biomass.

The data are shown in table 2, biomass activity was produced at a water soaked dates and date syrup concentration of 6.0 and 4.6 g/L respectively. Higher concentrations severely inhibited the biomass production. An elevated sugar concentration has been found to suppress pectinase production also in other microorganisms such as the fungus *Aspergillus japonicas* (*Al-Hooti et al., 2002;* Teixeira et al., 2000).

The effects of various nitrogen sources (initial concentration of 5 g/L) on final biomass concentration in a water soaked and date syrup based mineral medium are shown in table 3. $(NH_4)_2SO_4$ proved to be the best

Table 4. Effect of fermentation on biomass production of *B. megaterium* using water soaked at 8% $(NH_4)_2SO_4$, 30°C, 130 rpm

Time/hr	24	48	72	96	120
Water Soaked Biomass g/l	5.0	5.9	5.8	5.6	5.6

nitrogen source.

CONCLUSION

Optimized batch fermentations conducted in date syrup based medium using an isolated *Bacillus megaterium* yielded a biomass of 6.1 and 4.6 g/l for water soaked and date syrup respectively. This source was substantially greater than could be attained on media that used various other carbon sources. The optimal medium for producing the biomass was a mineral medium formulated with 8% of date syrup as the carbon source and 0.5 g/L $(NH_4)_2SO_4$as the nitrogen source. At optimal fermentation time of 48 hrs, at 30°C.Water soaked and date syrup can be used to inexpensively produce biomass in batch fermentations using *B. megaterium* as phosphorus solubilizing soil bacteria.

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