

Full Length Research Paper

Sonicated date syrup media preparation for microbial culture

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20% of the world's date exports are produced by Iran and much of these fruits are either damaged or has insufficient quality for human consumption. The proportion of the crop (35% of the total crops) that currently goes to waste can be turned into useful byproducts. Because of the high cost of commercial prepared culture media in Iran, it is envisioned that date waste can be used in the preparation of microbial culture media. In this study, various solidified date syrups were produced as culture media and the effect of date constituents with/without ultrasound waves irradiation were investigated using selected natural micro flora of date by agar dilution method and the results were compared with classical culture media containing PDA for fungi and PCA for bacterial cultures. The results showed that, extracted date syrup by ultrasound waves is an ideal media for enriching *Aspergillus* spp. and *Mucor* spp growth. Furthermore, it was demonstrated that date syrup containing media can also be used as a selective media due to its inhibition to certain bacteria and fungi. Hence, there is an economical benefit from the use of date syrup in the preparation of certain enrichment and selective microbiological media.

Key words: Date syrup, culture media, ultrasound, microbial.

INTRODUCTION

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.

Culture medium formulation (PBAC) agar was done by Cyzeska et al. (1981) for selective isolation and enumeration of gram-negative bacteria from ground meats. A mean recovery of 48% of total counts was obtained with PBAC agar whereas violet red bile agar overlay produced 28% of total counts. PBAC agar allowed the enumeration of 1.4 times as many gram-negative bacteria as violet red bile agar overlay. None of the eight strains of gram-positive bacteria and that of yeasts grew on PBAC agar (Cyzeska et al., 1981).

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. Correlation coefficients of DRBC versus PDA and DGA for recovering total mold and yeast counts from the composite of 14 foods indicated that, the three media were generally equivalent. Correlation coefficients for Petri film versus culture media were acceptable, although not as good as between culture media. Correlation coefficients of simulate versus DRBC, DGA, PDA and Petri film for recovering total yeasts and molds from a composite of 14 foods, demonstrated that there was no equivalence between the counts obtained by simulate, other culture media and Petri film, with significant differences observed for a majority of the foods analyzed (Taniwaki et al., 2002).

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. As the current research shows, the protein of raw potato wastes has been usable much more than steamed or solid-state wastes for microorganisms' growth (Qiyun and Liang, 2004).

A new marine medium was used by Vazquez et al. (2004) and a common commercial medium were evaluated for their effectiveness for promoting growth of different bacteria. Comparisons between the media were centered on the most important kinetic parameters of the corresponding cultures, that is, maximum biomass and specific growth rate, calculated by applying two widely accepted mathematical models (logistic and Gompertz equations) to measure data both in terms of dry weights and cell numbers. The parametric estimations allowed a classification of the results that demonstrated the effectiveness of all the media derived from fishery residues to meeting the proposed objectives. Growths were generally higher (up to 10 times in terms of cell numbers) than those from the common commercial medium, with the best results obtained from tuna (Vazquez et al., 2004).

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).

Use of feather-based culture media for the production of mosquitocidal bacteria was done by Poopathi and Abidha, (2007). Chicken feathers have been discarded in bulk as waste from poultry processing industries, poultry farms and shops, globally. They normally accumulate structural proteins (keratins) that are resistant to biodegradation. Considering the abundant supply of these feather wastes, they have successfully produced the biopesticides by culturing *Bacillus sphaericus* (Bs) and *Bacillus thuringiensis* serovar israelensis (Bti) strains to synthesize mosquitocidal toxins. Biochemical studies indicate that, the mosquitocidal spore/crystal toxins produced from the experimental culture medium (chicken feather waste medium, CFWM) are similar to that of conventional medium [nutrient yeast extract salt medium, (NYSM)]. The bacteria produced in these media (NYSM and CFWM) were bioassayed against the mosquito vectors (*Culex quinquefasciatus*, *Anopheles stephensi* and *Aedes aegypti*) and the toxic effect was found to be comparable. Cost-effective analysis indicates that the use of chicken feather waste as culture medium is highly economical for the industrial production of these mosquito pathogenic bacilli. This study is therefore, very important as it possesses the dual benefit of effective utilization of bio-organic waste materials from the environment and for the production of mosquitocidal biopesticides as well.

Two representative vegetable-based tryptic soy formulations were used to culture a range of bacteria and fungi by Cleland et al. (2007). Then the growth characteristics of them were compared with each other. All the representative of microorganisms grew well on the vegetable-based media and the media provided suitable recoveries of the organisms following simulated storage. Subtle phenotypic changes were observed between cultures grown on different media, but these did not significantly change the strain identification (Cleland et al., 2007).

Culture media formulations for industrial application were patented by Giovanni (2008). The invention related to formulations of culture mediums for the industrial development of liquid starter cultures, is characterized by a larger number of microbial cells per volume unit of fermentation medium than the one of traditional liquid. The method for preparing a culture medium includes the addition of a suitable amount basic neutralizing agent preferably to any traditional culture medium, depending on the microorganisms (Giovanni, 2008).

Potentials of cellulosic wastes in media formulation were investigated by Nwodo-Chinedu et al. (2009). Two agar media, Czapek-Dox and Sabouraud, were modified by substituting their carbon sources with cellulose, sawdust and sugarcane pulps. The modified Sabouraud's agar containing sawdust (Wood-Pep agar) and sugarcane pulps (Cane-Pepagar) yielded 84.4 – 100% of the maximum growth on Sabouraud's agar. Cellulose-containing media gave a lower level of growth (60.0 to 66.7%) of that obtained for the unmodified media

(Nwodo-Chinedu et al., 2009).

Nutritional effects of culture media on mycoplasma cell size and its effectiveness for removal by filtration was investigated by Folmsbee et al. (2010). The cell size of *Acholeplasma laidlawii* was measured after culture in various nutritional conditions using scanning electron microscopy. The maximum cell size changed, but the minimum cell size remained virtually unchanged and all tested nutritional conditions resulted in a population of cells smaller than 0.2 mm. Culture in tryptic soy broth (TSB) resulted in an apparent increase in the percentage of very small cells which was not reflected in increased penetration of non-retentive 0.2 mm rated filters.

A. laidlawii cultured in selected media formulations was used to challenge 0.2 mm rated filters using mycoplasma broth base as the carrier fluid. 0.2 mm rated filters was used as an analytical tool because *A. laidlawii* is known to penetrate 0.2 mm filters and the degrees of penetration can be compared. Culture of *A. laidlawii* in TSB resulted in cells that did not penetrate 0.2 mm rated filters to the same degree as cells cultured in other media such as mycoplasma broth or in TSB supplemented with 10% horse serum (Folmsbee et al., 2010). Because of the high cost of microbial culture media in Iran, researchers have tried to identify effective and inexpensive alternatives to commercially available media preparations. On the other hand, Iran currently produces 20% of the world's date exports. Reports suggest that, 35% of the total date crop is lost due to either damage or insufficient quality for human consumption. The proportion of the crop that currently goes to waste can be turned into useful by-products such as complex microbial culture media. Moreover, date waste can be accessed very inexpensively. In last stages of maturation of the date fruit, it typically contains 70.6 to 76.3% sugars, 1.9 to 3% proteins, 0.2 to 2.8% fat, 1.3% minerals, various vitamins and 24 to 25% water (Barreveld, 1993). So, these nutritious materials can be used for microbial growth in the form of complex microbial culture media which does not need the addition of sugar source.

MATERIALS AND METHODS

Date fruit micro flora preparation

Five unwashed date fruits (*Phoenix dactylifera* (L.), var *Kabkab*) were put in 200 ml sterile soy broth (SB) culture media and incubated on a laboratory shaker (95 rpm) at 37°C for 24 h; to increase the population of date fruit micro flora. Aliquots (0.5 ml) of the SB culture were inoculated on the surface of plate count agar and incubated at 37°C for 24 h. Microbial colonies were isolated and sub-cultured using SB and PCA as reported earlier. The procedure was carried out in duplicate for each isolate studied. Ultimately, the selected colonies were characterized by morphological and biochemical markers as date fruit micro flora.

Culture media preparation from date fruit

For culturing date fruit micro flora, pitted date fruit (Kabkab date)

were mixed with water in 1:3 ratios by classical mixer and equilibrated at room temperature for 5 min. The samples were irradiated by ultrasound (XL 2020 Model, 20 KHz Frequency) waves with 150 W intensity for 10 min. After diluting the mixture with water at a ratio of 1:9, it was twice filtered; first with filter cloth (for removing date particles) and then by millipore filters paper [0.2 μ (Sartorius)] using a laboratory vacuum pump for cold sterilization of the date syrup. Then, the exudates fraction from the millipore filter was used for culture media base. The date-syrup formulated culture media was solidified using sterile condensed agar (MERCK) solution. Then, the prepared culture media was distributed in sterile plates and allowed to solidify. Finally, the media was inoculated with the isolated micro flora of the date fruit (five replicates /examination) according to agar dilution method as a recommended standard method (Brown, 1994).

Results produced from mean of 5 data sets were compared with classic culture media containing PDA for fungi and plate count agar (PCA) for bacterial cultures. The results were recorded after the incubation interval.

RESULTS

Date syrup components effect on isolated micro flora of date fruit

This was obtained from mean of 5 replicated microbial count of date fruit micro flora which were cultured in classic media (PDA for fungi and PCA for bacterial cultures) and date syrup media prepared with or without irradiation exposure of ultrasound waves.

Fungi

Aspergillus sp

The results showed that date fruit components induced *Aspergillus* growth 1.3 times more than (PDA) (Table 1). These results suggest that *Aspergillus* is an important fungus for date fruit infection and spoilage; date fruit had more suitable ingredients for *Aspergillus* growth as a culture media than PDA. Therefore, it appears that inexpensive date-based media can be used as an effective alternative to commercially prepared media for cultivation of *Aspergillus*.

Mucor

Date syrup components helped *Mucor* growth 2.16 times more than PDA. This enhanced growth was more pronounced when extracted by ultrasound such that it equaled 2.87 times yields observed with PDA (Table 1). Hence, it is concluded that *Mucor* is a major infection factor for date and date products; date syrup media can be used for the enrichment of *Mucor* culture media in microbiological analysis. Application of ultrasonic irradiation to date syrup media had noticeable effect on the growth rate of *Mucor* (Figure 1).

Table 1. Microbial count in control and date syrup media /sonicated date syrup.

Microbial type	PDA/PCA (count/ml)	Date syrup (count/ml)	Sonicated date syrup (count/ml)
<i>Aspergillus</i>	300	400	400
<i>Mucor</i>	80	173	230
<i>Gilmaniella</i>	200	100	15
<i>Saccharomyces rouxii</i>	288	298	269
<i>Labrintula</i>	10	10	12
<i>Zygosaccharomyces</i>	10	18	18
<i>Bacillus cereus</i>	340	40	38
<i>Staphylococcus</i>	480	6	6
<i>E. coli</i>	430	20	12

Mean of 5 replicated microbial count of date fruit micro flora cultured; classic media (PDA for fungi and PCA for bacterial cultures). Date syrup media prepared with or without irradiation exposure of ultrasound waves.

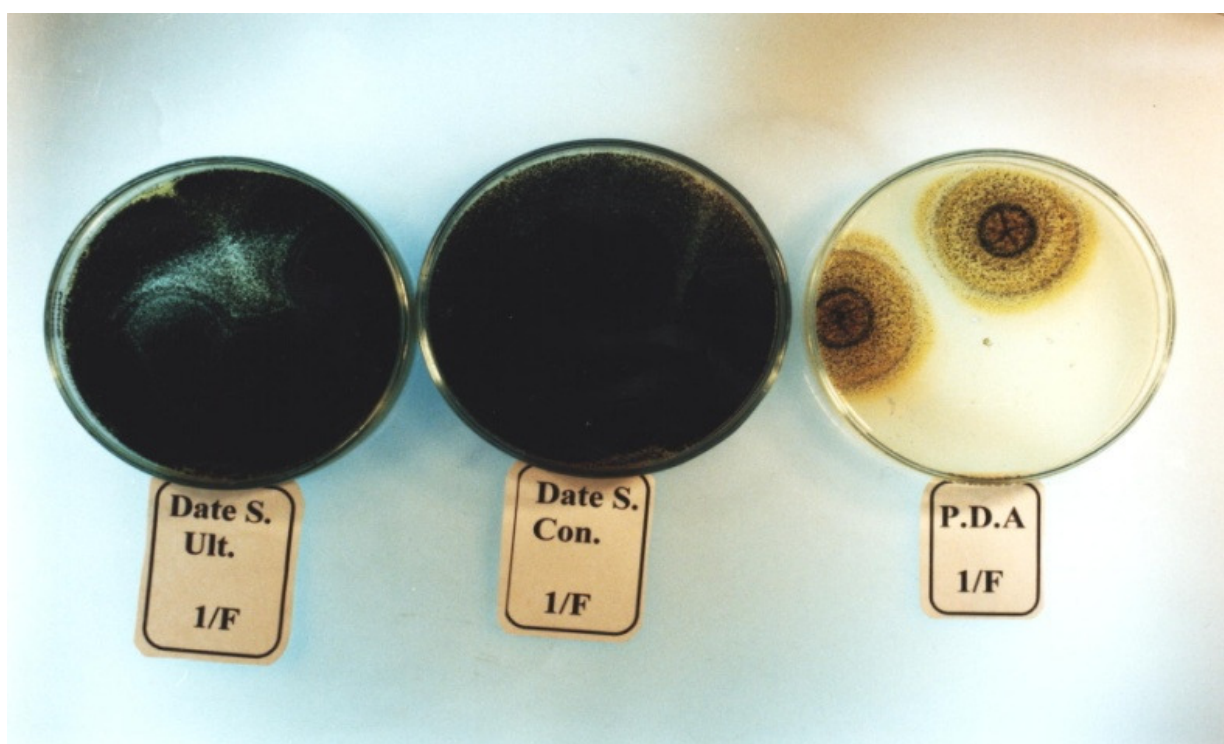


Figure 1. Date culture media prepared with/without ultrasonication and its effect on *Mucor* growth in comparison with culturing in classic media (PDA).

***Gilmaniella* sp.**

The culturing results showed that *Gilmaniella* sp. was susceptible to date fruit components. *Gilmaniella* populations were decreased by 50% in date-base media when compared to PDA. Moreover, when sonicated date syrup media was used, there was 92.5% decrease in the organism population (Table 1). It was concluded that, date fruit has components with antimicrobial effect which restricted the growth of *Gilmaniella* especially when date syrup was irradiated by ultrasonic device.

Saccharomyces rouxii

Date syrup media resulted in the growth of *Saccharomyces rouxii* at level comparable to those achieved when using commercially prepared PDA (Table 1). So, it can be used as a substitute for classical product.

Labrintula

The results showed that date syrup and PDA had a

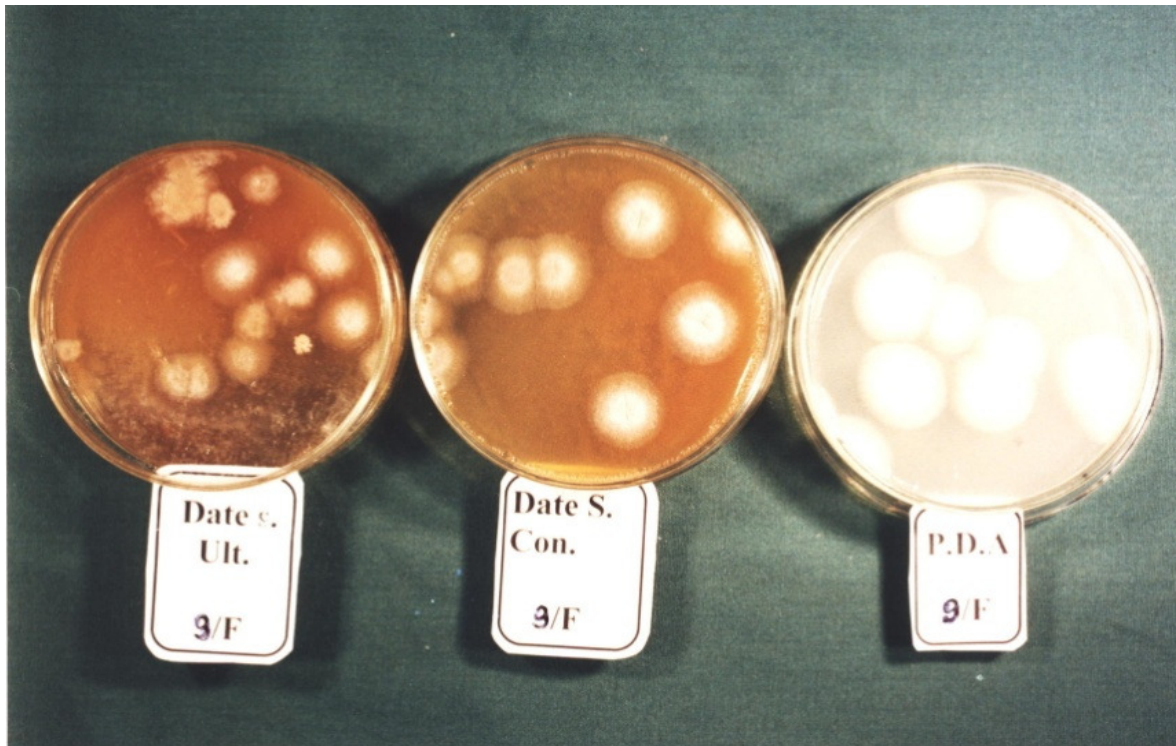


Figure 2. Date culture media prepared with/without ultrasonication and its effect on *Labrintula* growth in comparison with culturing in classic media (PDA).

similar effect on the growth of *Labrintula* (Table 1) and ultrasonication did not have any additional effects. The population of this organism was the same in both culture media. Therefore indicating that, *Labrintula* was resistant to components of date fruit syrup. Date syrup media can be used as a selective media for this microorganism (Figure 2).

***Zygosaccharomyces* sp.**

This is a yeast tolerated date fruit media. *Zygosaccharomyces* increased the growth by 1.8 times when compared to PDA as shown in Table 1. Similar results are also reported for the response of *Saccharomyces rouxii* growth in date fruit media (Figure 3). This resulted due to cheap price of date fruit in Iran. Its media can be preferred for both yeasts.

Bacteria

Gram positive bacteria

Bacillus cereus

Bacillus cereus growth was decreased in the date syrup media by almost 88% in comparison with PCA media

(Table 1). Although, the application of ultrasonic irradiation to date syrup media had no noticeable effect on the decreased growth rate (Figure 4).

***Staphylococcus* sp.**

This gram positive bacterium was susceptible to the date syrup media culture components (Table 1) and its population was decreased approximately by 98% after the incubation period (Figure 5).

Gram negative bacteria

Escherichia coli

This gram negative bacteria's population, was somewhat restricted in its growth by the date components (Table 1). The observed levels of inhibition were on the order of about 49%. However, when date syrup media was sonicated, its effectiveness in inhibiting this organism increased to 81% (Figure 6).

DISCUSSION

As mentioned earlier, for novel extraction of date fruit

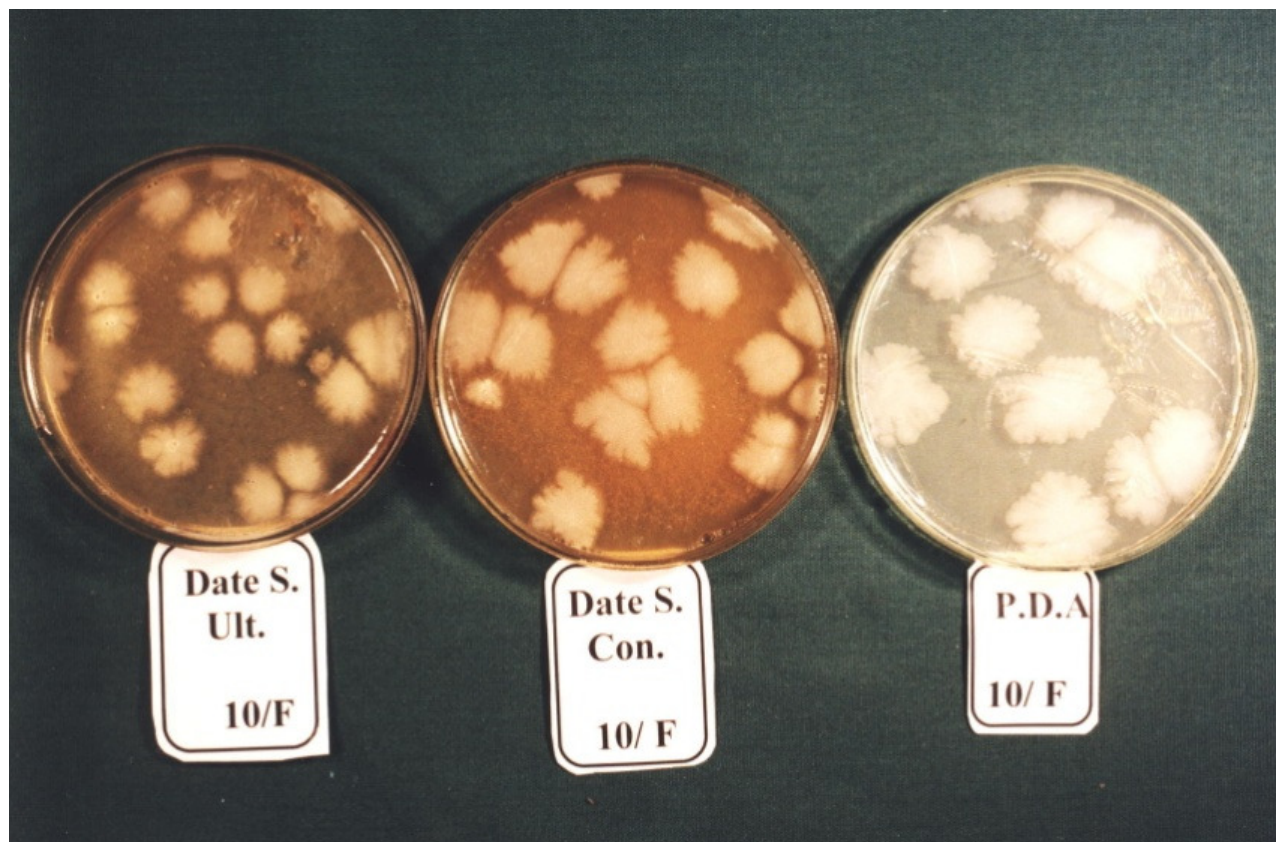


Figure 3. Date culture media effect on *Zygosaccharomyces* sp. growth in comparison with culturing in classic media (PDA).

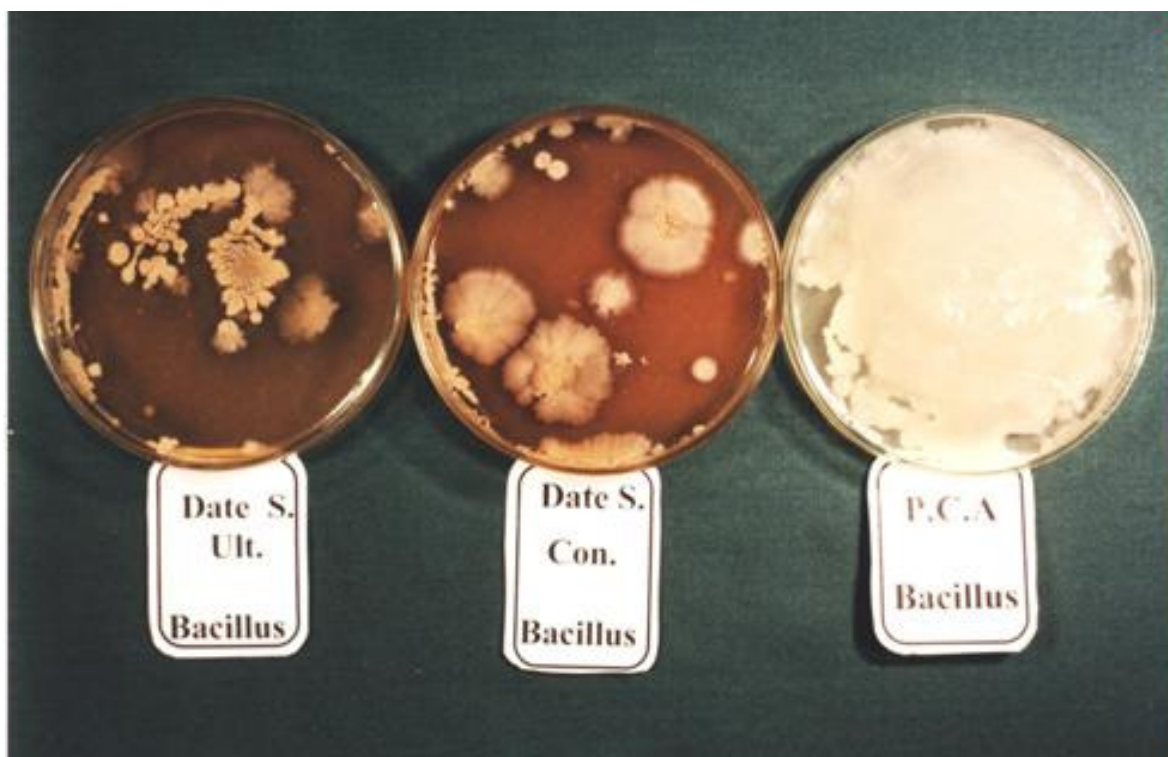


Figure 4. Date culture media effect on *Bacillus cereus* growth in comparison with culturing in classic media (PCA).

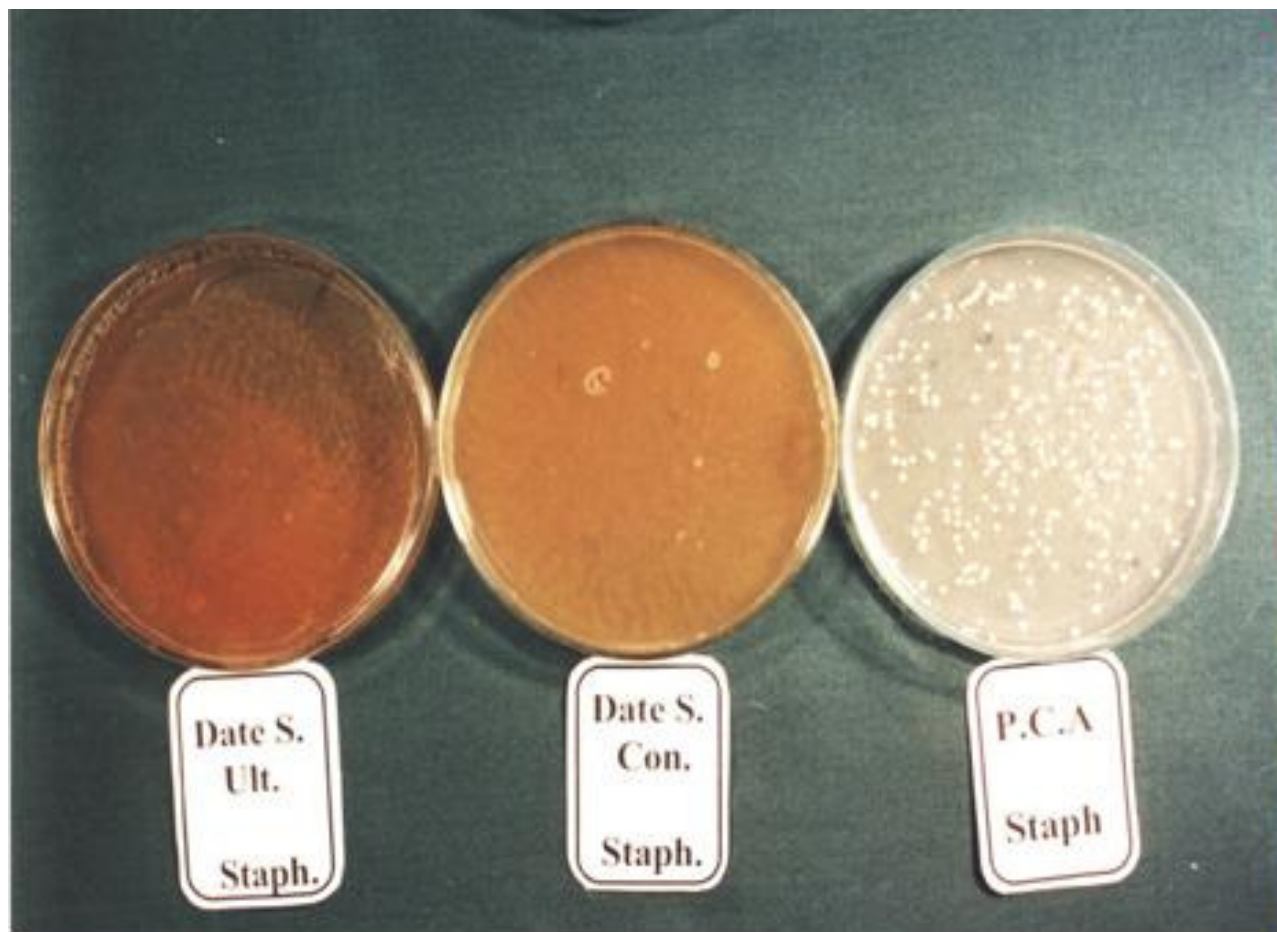


Figure 5. Date culture media effect on *Staphylococcus sp.* growth in comparison with culturing in classic media (PCA).

components, the samples were irradiated by ultrasound (XL 2020 Model, 20 KHz Frequency) waves with 150 W intensity for 10 min because according to the previous investigation of Entezari et al. (2004) ultrasound waves improved date components extraction due to its mechanical effect. So, it can spread in liquids and vibrates fluid molecules in the direction of its own vibration, so it has the associated mass transfer properties. Also, mechanical effect of ultrasound wave originates from the collapse of cavitations' bubbles within the treated substrate when adequate intensity of ultrasonic waves produces cavities. Cavitations and its explosion releases bounded materials and accelerate the extraction of intracellular components (Entezari et al., 2004). Also, all of the investigations confirmed the positive effect of ultrasound on the extraction of various materials (Beck, 2007; Cyzeska et al., 1981; Entezari et al., 2004). Similar results by ultrasonication of date and water mixture were obtained. As the figures shows, there are some differences in the microbial count results of the two procedures (date culture media preparation with/without ultrasonication due to different effect of extraction manner of date fruit materials for example in *Mucor*).

Date fruit materials in some samples, also caused negative or positive effect on the microbial growth. It showed different required materials for various microbial growths, so date culture media can be used as selective media for *Aspergillus* and *Mucor* as culture medium formulation (PBAC) agar was done by Cyzeska et al. (1981), for selective isolation and enumeration of gram-negative bacteria from ground meats.

Since this work aimed at using date syrup media, there are many investigations for substituting cheap 'media' instead of classical culture 'media' like plant or animal products wastes. As such, studies that used potato processing waste as a fermentation substrate for the production of single cell proteins (SCP) was used in the supplementation of animal feeds (Qiyun and Liang, 2004). The conventional medium palm kernel agar (PKA) was used for the recovery of aflatoxigenic fungi from mixed cultures, the detection of aflatoxigenic fungi and direct visual determination of aflatoxins in agricultural commodities (Atanda et al., 2006). However, the modified Sabouraud's agar containing sugarcane pulps yielded 100% of the maximum growth on Sabouraud's agar (Nwodo-Chinedu et al., 2009).

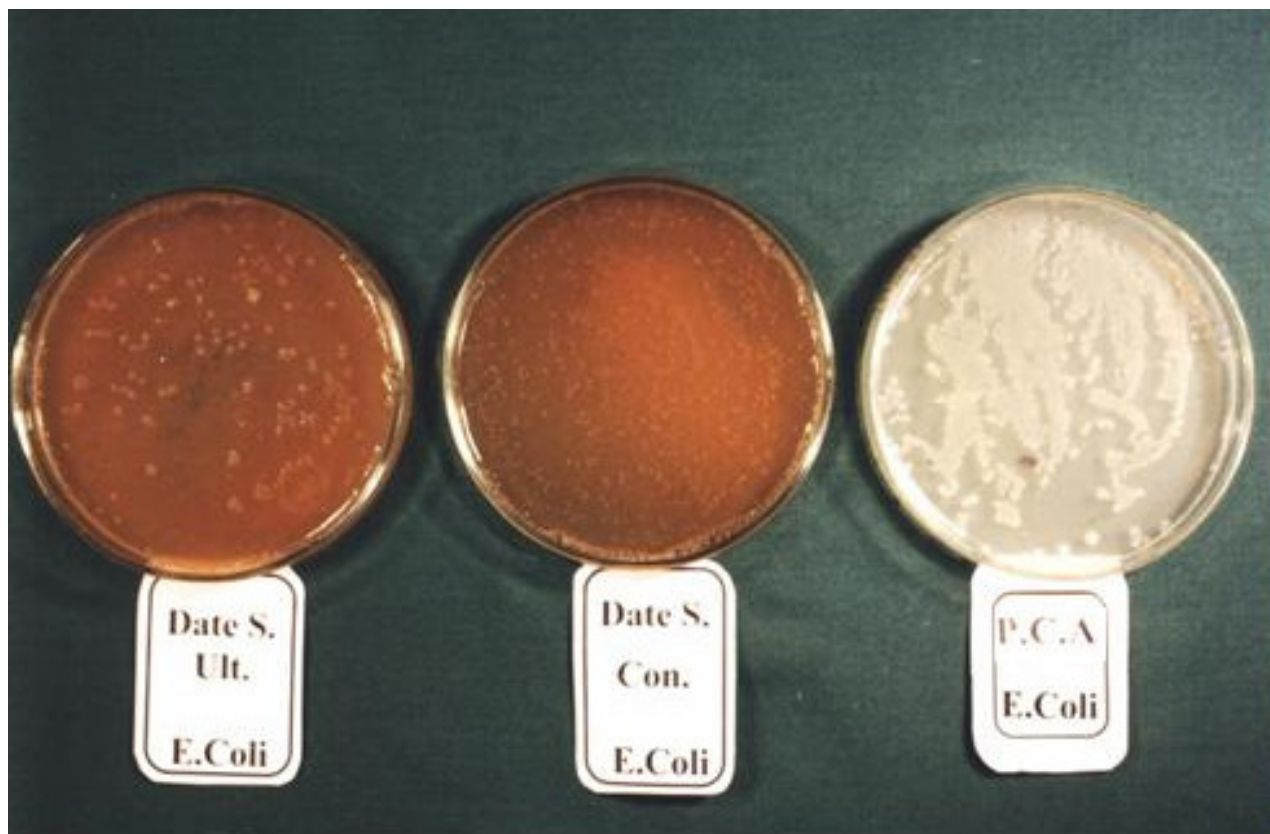


Figure 6. Date culture media effect on *E. coli* growth in comparison with culturing in classic media (PCA).

The use of feather-based culture media for the production of mosquitocidal bacteria (Poopathi and Abidha, 2007), a new marine medium and a common commercial medium was evaluated for their effectiveness for promoting growth of different bacteria (Vazquez et al., 2004). In comparison, the above media formulation; date syrup media helped *Mucor*, *Zygosaccharomyces* and *Aspergillus* growth, 2.16, 1.8 and 1.3 times respectively, more than classical culture media.

In comparison with the commercially prepared media, date culture media has naturally balanced amount of protein and since its production is only by mixing and ultrasound irradiation, it is not heated; therefore the materials which may restrict microbial growth rate are not altered as seen in Qiyun and Liang (2004). The protein of raw potato wastes has been used much more than steamed or solid-state wastes for microorganisms' growth (Qiyun and Liang, 2004).

Conclusion

Date culture media that is the focus of this work have been shown to possess sufficient amounts of nutrients for support of the growth of microorganisms such as *Aspergillus* and *Mucor*. It was also shown that, date

extracts are capable of suppressing the growth of other fungi and bacteria. For example, the growth of the fungus *Gilmaniella* and the spore forming bacteria *Bacillus* were suppressed or inhibited. It is postulated that the suppression of the growth of some susceptible microorganisms may be due to the phenol compounds existing in date fruit components.

Sonication of date fruit mixture was shown to accelerate the extraction of nutritive materials necessary for promoting growth of the fungi used in this study. Culture media prepared using sonicated date syrup, can play an important role in the formulation of both selective and enrichment culture media for fungi such as *Mucor*, *Aspergillus* and *Saccharomyces* spp.

This work has shown that, date fruit and its waste products can be used efficaciously and economically for the cultivation of the fungi that were reported in this work. Moreover, due to equal effect of PDA and date culture media on the growth of *Saccharomyces*, it is suggested that, date waste containing media can be used for beer-brewing technology.

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