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Antioxidant, phenolic compounds and antimicrobial activity of yoghurt and bioyoghurt fortified with sedr honey

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ABSTRACT

The aim of this study was to investigate the effect of bioactive compounds such as phenolic compounds, antioxidants, organic acids, carotenoids, and vitamins of sedr honey at different concentrations 5,10 and 15% on activity and vitality of probiotics and lactic acid bacteria during fermentation as well as, total colony counts, *aerobic& anaerobic bacteria , Str. thermophillus, Lb. bulgaricus ,Bifidobacteria count, Coliform ,yeasts and molds counts* affecting the prolongation of the yoghurt shelf life till second week of storage at 6±2°C. Bioyoghurt (3% probiotic ABT-5 cultures) and yoghurt (made by traditional yoghurt culture) were held at 6±2°C for 14 days. The titratable acidity and total solids of yoghurt & bioyoghurt were increased with increasing sedr honey concentrations and storage period. Also, as the storage period progressed, the fat, total protein and ash contents of yoghurt gradually decreased for control and all treatments. The total plate, *B. bifidum* counts and *Str. thermophilus* were increased in all treatments till the end of storage period. Bioyoghurt made with 5% sedr honey gained the highest of overall scores. Supplementation of yoghurt with levels of honey affected the viability of probiotic bacteria. Thus, it can be concluded that *Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus* viable counts increased with 10% honey addition as compared to 5 %, 10%, 15%, and 20% honey.

Key Words: Sedr honey, antioxidant, phenolic compounds, antimicrobial activity, chemical, microbiological composition and sensory properties, Yoghurt and Bioyoghurt.

INTRODUCTION

Yoghurt is one of the most popular fermented milk products worldwide because it has many health benefits such as improving lactose intolerance, reducing risk of certain cancers, anticholesterolaemic effects, prevention of genital and urinary tract infections (Savadogo *et al.*, 2006) and other health attributes associated with probiotic bacteria (Mckinley, 2005; Sibel Silici *et al.*, 2010). Several therapeutic and medicinal effects such as antibacterial, antimutagenic, antiproliferative, hepatoprotective, hypoglycemic, and antioxidant effects have been ascribed to honey through last years (Poorani *et al.*, 2012). It has been observed that molds, yeasts and bacterial spores can be present in honey at low levels, but vegetative bacteria generally are not found. Besides its sugar composition, honey consists of a number of bioactive compounds such as phenolic compounds, flavonoids, carotenoid-like derivatives, organic acids, Maillard reaction products, catalase, ascorbic acid, and other compounds which function as antioxidants (Bogdanov *et al.*, 2008). Several therapeutic and medicinal effects such as antibacterial, antimutagenic, antiproliferative, hepatoprotective, hypoglycemic, and antioxidant effects have been ascribed to honey through last years (Tamime and Robinson, 1985) and (Ammar, et al . 2015) .The high osmolarity and acidity of honey are among the physical characteristics that contribute to its antibacterial activity. Hydrogen peroxide, volatiles, organic acids, flavonoids, beeswax, nectar, pollen and propolis are important chemical factors that provide antibacterial properties to honey (Olaitan, *et al.*, 2007). Finally, honey could be used as a sweetener and prebiotic in order to improve fermentative aptitudes of bifidobacteria in desirable flavor mix probiotic product and with a relatively stable shelf life (Riazi and Ziar 2012). Inhibitory properties of honey against pathogens such as *Bacillus cereus, Listeria monocytogenes, Escherichia coli, Mycobacterium tuberculosis, Salmonella typhi, Salmonella typhimurium, Shigella* spp., *Staphylococcus aureus, Vibrio cholera*, number of Gram positive & Gram

negative bacteria, aerobic & anaerobic bacteria, *Candida albicans* and *Helicobacter pylori have been demonstrated* (Molan, 1992).Honey has been found to contain significant antioxidant activity attributed to glucose oxidase, catalase, ascorbic acid, flavonoids, phenolic acids, carotenoid derivatives, organic acids, Maillard reaction products, amino acids, proteins. Antioxidants bind to the Transcription Factors and prevent harmful effects as cancer, cardiovascular diseases, inflammatory disorders, neurological degeneration, wound healing, infectious diseases and aging. (Abeshu and Geleta 2016) .

Microbial inhibition of honey has been attributed to its low pH, osmolority, acidity and chemical factors such as hydrogen -peroxide, volatiles, propolis and unidentified substances from certain floral sources (Ndip et al.2007), in addition to the presence of enzymes such as glucose oxidase, catalase, and lysozyme. Compounds such as 3,5-dimethoxy-4-hydroxybenzoic acid (syringic acid), methyl- 3,4,5-trimethoxybenzoate, and 3,4,5- trimethoxybenzoic acid and methyl 3,5-dimethoxy-4-hydroxybenzoate (methyl syringate) have been isolated by (Ediriweera and Premarathna 2012) .Darker colored honeys were generally inhibitorier than light colored honeys. Darker honeys also contained higher antioxidant power (Taormina *et al.*, 2001). Mycobacteria bovis did not grow in culture media containing10 % and 20 % honey while it grew in culture media containing 5, 2.5% and 1% honey. Also heating to 80"C for 1 hour decreased antimicrobial activity of both new and stored honey. Storage of honey for 5 years decreased its antimicrobial activity (Al-Waili, 2004).

The objective of this study was to develop a desirable healthy yoghurt and bioyoghurt using sedr honey as a sweetener and natural prebiotic in lieu of sucrose and by incorporating ABT-5 cultures. Thus, the growth of bacteria and their pH changes in milk were studied until coagulation, also the effect of adding these materials on some chemical, microbiological and quality characteristics during storage for 2 weeks at 6 ±2°C. The effect of bioactive compounds such as phenolic compounds, antioxidants, organic acids, carotenoids, and some vitamins of sedr honey at different concentrations 0,5,10 and 15% on activity and vitality of probiotics and lactic acid bacteria during fermentation as well as, yeasts and molds count affecting the prolongation of the yoghurt and bioyoghurt shelf life. Also bioactive compounds of the most favorite treatments at sensory properties had been studied during the storage period.

MATERIALS AND METHODS

Procurement of raw material

Buffalo's milk was obtained from the Collection Milk Center in Arab EL Awamer, Assuit, Egypt. *Lactobacillus delbrueckii ssp. bulgaricus* LB340 and *Streptococcus thermophilus* were obtained from Dairy Enzymes Applications, Danisco, France. Probiotic cultures were provided by Chr. Hansen, contains *ABT-5 cultures* (*Lactobacillus acidophilus, Bifidobacterium bifidum and Streptococcus thermophilus*). Sedr honey was obtained from Local Markets, Assuit, Egypt.

Manufacturing of yogurt

Yoghurt and bioyoghurt were prepared according to (Tamime and Robinson, 1985) as below

Milk

↓

Heat treatment

(At 90±1ºC for 5 min and cooling to 40-41°C)

↓

Inoculated with 2% active starter cultures of *lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus*

(or Inoculated with 3% active starter cultures of *ABT-5 Cultures in case of plain bioyoghurt)*

↓

Distributed into plastic cups

↓

Incubated at 42°C until complete coagulation

↓

Held at 6±2°C for 14 days

In case of Sedr Honey Yoghurt and bioyoghurt , Buffalo's milk was divided into four parts. The first part was set as control and then Sedr honey was added at 5, 10 and 15% as the following steps shortly:

Milk

↓

Divided into four equal parts

↓

Heat treatment

(At 90±1ºC for 5 min and cooling to 40-41°C)

↓

Adding of sedr honey

(T_1, T_2, T_3 and T_4)

↓

Inoculated with 2% active starter cultures of *lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophilus*

(or Inoculated with 3% active starter cultures of *ABT-5 Cultures in case of bioyoghurt)*

↓

Distributed into plastic cups

↓

Incubated at 42°C until complete coagulation

↓

Held at 6±2°C for 14 days

T_1: Control; T_2: Adding 5% sedr honey; T_3: Adding 10% sedr honey; T_4: Adding 15% sedr honey.

Manufacture of Sedr Honey Bioyoghurt:

Buffalo's milk was divided into four parts. The first part was set as control and then sedr honey was added at 5, 10 and 15% as the following steps shortly:

Milk

↓

Divided into four equal parts

↓

Heat treatment

(At 90±1ºC for 5 min and cooling to 40-41°C)

↓

Adding of sedr honey

(T_1, T_2, T3 and T_4)

↓

Inoculated with 3% active starter cultures of ABT-5 Cultures

↓

Distributed into plastic cups

↓

Incubated at 42°C until complete coagulation

↓

Held at 6±2°C for 14 days

METHODS OF ANALYSIS:

Chemical Analysis

The titratable acidity in yoghurt and bioyoghurt in this study was measured according to the method as described in A.O.A.C. (2000). On the other hand titratable acidity in honey was measured according to A.O.A.C. (2000) by using sodium hydroxide N/0.05 and pH meter and calculated as milliquivalent/kg:

Free acidity = (ml0.05N. NaOH from buret - mL blank) ×50/g sample

Lactone = (10.00 -ml0.05N. HCL from buret) ×50/g sample

Total acidity = Free acidity + Lactone.

Fat percentage was determined by using Gerber in yoghurt and Soxelt method in honey as described by Ling, (1963). The total protein content was determined by using the Kjeldahel method according to A.O.A.C. (2000). The total Solids content were measured according to A.O.A.C. (2000)**.** The ash contents were measured according to A.O.A.C. (2000).Some major elements as well as Mg-Na-K-Mn-Fe-Ca-Zn and Cu were measured according to the method as described by James (1995).The dilutions were applied by using the atomic absorption spectrophotometer to estimate the levels of Mg-Na-K-Mn-Fe-Ca-Zn and Cu (ppm) using atomic absorption spectrophotometer(3300 PERKIN ELMER). Vitamins as well as A, C, D, E and Phenols were determined by High-Performance Liquid Chromatography (HPLC), according to Jayaprakasha *et al*. (2003). Total sugars had been measured according to Anthron method (Hedge and Hofreiter, 1962) by using spectro-photometer (Uvi-line 9400, Germany) in Agricultural Research Center, Egypt.

Microbiological Analysis

Preparations in both samples of yoghurt and bioyoghurt and dilutions for the microbiological examinations were carried out according to FIL/IDF 66 (1971). Total bacterial count (TBC) in both yoghurt and bioyoghurt was determined by using the standard plate count technique as described by (Marshall, 2004). Appropriate dilutions of the samples were plated in duplicate on nutrient agar medium as

described in Difco manual, (1998). Detection of coliform bacteria was estimated according to FIL/IDF 65 (1971). Triple tubes were used in MacConky Broth medium and incubated at 37°C for 48 h. Counts of aerobic and anaerobic bacteria were carried out according to A.O.A.C. (2000). M17 agar was used for the enumeration of *Streptococcus thermophilus*, MRS for *Lactobacillus delbrueckii ssp. bulgaricus* and modified MRS for *Bifidobacterium bifidum* as described by Dave and Shah, (1996). Counts of yeasts and moulds were carried out according to the International Dairy Federation FIL/IDF 67 (1971).

Organoleptic Properties

The organoleptic properties of yoghurt and bioyoghurt were assessed by a panel test of 10 persons of stuff members of Agriculture Research Center, Assuit as fresh, 3, 7 and 14 days of storage period at 6±2°C according to the scheme described by Badawi *et al.* (2008).

Statistical Analysis

Results were evaluated statistically using the soft program, the SAS system for windows, release 8.02 TS level 02M0, SAS Institute Inc., Cary, NC, USA (SAS, 1999).

RESULTS AND DISCUSSIONS

Sedr honey

The chemical parameters of sedr honey are illustrated in (Table 1). And it is clear that the acidity was 50.00 meq/kg, fat content 0.34%, protein 2.30%, moisture 18.48 % total solids 81.52, ash 0.64% and total sugars 70.17 %. These results are in partly agreement with those of Oyeleke *et al.* (2010) who found that total titratable acidity (32.6%), fat content (1.5%), protein content (0.88%), moisture content (25.22%), ash content (1.67%) and carbohydrates (69.53%) for Nigerian honey. The most abundant metal in honey was K (93.55 ppm). Other major metals present in honey were Ca (second most common) (35.00), Na (34.25), Mg (18.60), Fe (0.71) Zn (0.52), Cu (0.52 ppm) and Mn (0.033). These results are in partly agreement with (Nigussie *et al.*, 2012). As for the antioxidants content, vitamin C was the most abundant in honey 22.214 ppm. On the other hand, vitamin A and D was found in a lower value. These results are in partly agreement with those who obtained by (Islam *et al.*, 2014). The concentrations of phenolic compounds in sedr honey are given in Table 2. From this table it could be observed that the amount of Cinnamic, Ellagic, Ferulic, Vanillic, Catecol, Salycilic, and Protocatchuic were detected. Also Coumarin, Caffeine, Caffeic, Chlorogenic, Catechein and Hydroxy tyrosol was not detected in honey samples. These results are matching with (Hussein et al., 2011).

Yoghurt Fortified with Sedr Honey

Chemical composition of yoghurt fortified with sedr honey:

The titratable acidity in yoghurt during storage period were increasing as shown in Table 3. Gradually with the increasing of honey concentration add from 0.81 % (control) to 1.47 (T3) at fresh time .These values are agreement with Ghadge *et al.* (2008), they showed that the acidity of fortified yoghurt increases as the concentration of fortification increased because honey contains organic acids which increase the acidity. That means, acidity of ingredients affects acidity of yoghurt. As expected, the mean value of T.A of fresh treatments were significantly lower than those stored which are due to the partial fermentation of lactose also the acidity (lactic acid content) goes on increasing with the progress of the storage period .These results partly compatible with those of Chick et al. (2001) and (Stijepic *et al.*, 2012) who found that honey was not inhibitory to *S. thermophilus*, *L. delbrueckii* ssp.*bulgaricus*, *L. acidophilus*,or *B. bifidum* at a level of 5% with honey enhancing lactic acid production by *Bifidobacteria*.

Results of fat content in honey yoghurt increased as well as increase the percentage of honey and the highest fat percentage score was recorded in T3 (4.25 %) at the end of storage period (14 day). The values of fat content intend to increase with the progress of the storage period. These results are partly in agreement with those obtained by (Stijepic *et al.*, 2012) who observed that the presence of bee honey had insignificant influence on fat content in the resultant yoghurt, while cold storage significantly ($p \leq 0.05$) increased the mean values of fat. On the other hand Ghadge *et al.* (2008) and Rashid and Thakur (2012) observed a decreasing in fat percentage with increase in concentration of honey yoghurt. The results of protein content increased as well as increase the percentage of honey, because honey contains considerable protein content 2.30% these finding is nearly coincided with the results of Rashid and Thakur (2012). The highest protein percentage score was recorded during storage period were in T3 (4.45%) at day 3. Generally the total solids increased as well as increase the percentage of honey because honey contains higher amounts of total solids (81.52%) The highest total solids percentage score was recorded in

T3 (19.58%) at day 14. These results are in agreement with the results obtained by (Wedad and Owayss, 2009). The results of ash content decreased as well as increase the percentage of honey, because sedr honey contains low ash content (0.64%). The highest ash content percentage score was recorded in control (0.88%) at initiation and during storage decreased gradually till the end of storage (0.69%).These results are in agreement with the results obtained by Wedad and Owayss (2009).

Bacteriological analysis of yoghurt fortified with sedr honey

The results (Table 4) showed total bacterial count (TBC) increased and reaches their maximum counts at the end of storage period. These results are partly in agreement with Abd-Elsalam *et al*. (2011) who found that the TBC count for all treatments increased through the first two week of storage period, then declined at the third week, although the control showed the lowest count during the storage period, whereas the treatments with the highest count was that of supplemented with honey. Counts of *Str. thermophillus and Lb. bulgaricus* bacteria increased up to the end of storage period. These results might be due to the effect of cold storage and acidity development on bacterial growth. These results are in agreement with those reported by Abd-Elsalam *et al*. (2011) they found that counts of *Str. thermophillus and Lb. bulgaricus* increased up to the second week of storage period and then decreased gradually in all treatments up to the end of storage period. Also Varga (2006) added that the presence of honey at 1.0% to 5.0% (w/v) did not significantly influence (P>0.05) the viability of characteristic microorganisms (i.e., *Streptococcus thermophiles* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) in yoghurt during storage at 4 °C. The counts of yeasts and moulds appeared at the 7 days of storage period at the most of treatments, this might be due to the severe of heat treatments, the microbial load of added honey and the post contamination after manufacture, and decreased at the end of storage period might due to the post contamination after manufacture.

These results are in agreement with El-Nagar and Brennan (2001) who reported similar results. Likewise, the coliform bacteria counts were not detected in both fresh as well as at the termination of storage for all treatments. This might be due to severe of heat treatments of milk and the role of lactic acid bacteria in preservation of the products which associated with their ability to produce a range of antimicrobial compounds and results are in line with findings of of Rashid and Thakur (2012). Furthermore, the aerobic and anaerobic bacteria counts were not detected in all treatment except (T3) at 3 day to the end of storage period this might be due to the microbial load of added honey and the post contamination after manufacture. And at the end of storage period in all treatments this might be due to the severe of heat treatments of milk and the role of lactic acid bacteria in preservation of the products which associated with their ability to produce a range of antimicrobial compounds. Nearly the same results were recorded by Abdel Fattah (2006) who mentioned that the aerobic bacteria counts were not detected till the 5th day of storage time.

Organoleptic properties of yoghurt fortified with sedr honey

The organoleptic evaluation indicated that all scores of treatments increased up to the end of storage period with significant differences.

Bioyoghurt Fortified with Sedr Honey

Chemical composition of bioyoghurt fortified with sedr honey

Table 6 shows that the titratable acidity content of the resultant bioyoghurt was affected by the percentage of added herbs honey and storage period. The titratable acidity content of bioyoghurt fortified with sedr honey was increased significantly (p<0.05) with the progressing of storage period in all treatments (0.7% in control - 1.4% in T3) at fresh time. These results are in harmony with those of Chick *et al*. (2001) who mentioned that the organic acids production was enhanced when *Bifidobacteria* were grown in the presence of honey. The values of fat content in honey bioyoghurt increased as well as the increasing in the percentage of honey (3.93 in control - 4.4 in T3) at fresh time. These results are in agreement with those of (Farag *et al*., 2007). The results of protein content increased with the increasing in the percentage of honey (4.07% in control - 4.5 % in T3) at fresh time. Because honey contains considerable protein content 2.30% these finding is nearly coincided with the results of (Akalin, 1996).

Generally, the total solids increased with the increasing in the percentage of honey (11.00 % in control - 16.97% in T3) at fresh time because honey contains higher amounts of total solids. These results are in agreement with those of (Akalin *et al*., 2004). The results of ash content decreased significantly with the increasing of honey (0.78 % in control - 0.68 % in T3) at fresh time. The variations in the ash content of

bioyoghurt were found to be significant ($P < 0.05$) during storage and results are in harmony with Ammar *et al*. (2015).

Table 1. Chemical composition of sedr honey

Parameters	Values
Acidity (meq/kg)	50.00
Moisture (%)	18.48
Total solids (%)	81.52
Total sugars (%)	70.17
Total protein (%)	2.30
Fat (%)	0.34
Ash (%)	0.64
Mg(ppm)	18.60
Na(ppm)	34.25
K(ppm)	93.55
Mn(ppm)	0.033
Fe(ppm)	0.71
Ca(ppm)	35.00
Zn(ppm)	0.52
Cu(ppm)	0.52
Retinol (ppm)	0.347
Calciferol (ppm)	5.07
Ascorbic acid (ppm)	22.21

Bacteriological analysis of bioyoghurt fortified with sedr honey

The results in Table 7 shows that the total plate count, *Str. thermophilus* and *B. bifidum* counts of bioyoghurt were affected by the addition of honey and storage periods. The total viable bacterial count increased during storage period .However, the total bacterial counts of bioyoghurt containing honey were higher than that of the control in fresh and at the end of the storage period. Similar results are obtained by (Abd-Elsalam *et al*., 2011). Counts of *Str. thermophillus* bacteria increased till the end of storage period. These results might be due to the effect of cold storage and acidity development by bacterial growth. These results are in partly agreement with those of Riazi and ziar (2012). *Bifidobacteria* increased gradually at the most of honey bioyoghurt treatments up to the end of storage period. Abd- El-Salam *et al*. (2011) stated that supplementation of yoghurt with honey and *B. lactis* improved growth of bacterial starter.

Table 2. Phenols content (ppm) of sedr honey

Phenolic compounds	Nil
Oleuropein	3.6119
Cinnamic	Nil
Coumarin	0.00278
Ellagic	Nil
Benzoic	0.00121
Salycilic	2.1282
Ferulic	Nil
Caffeine	1.0418
Vanillic	Nil
Caffeic	Nil
Chlorogenic	14.5704
Catecol	Nil
Catechein	11.1765
Protocatchuic	Nil
Hydroxy tyrosol	

The counts of yeasts and moulds were not detected till the 7 days of storage period at the most of treatments, this might be due to the severe of heat treatments, the microbial load of added honey and the post contamination after manufacture and decreased at the end of storage period might due to the post contamination after manufacture. These results are partly in agreement with those of Abd- El-Salam *et al*. (2011). On the other hand, the coliform bacteria counts were not detected in both fresh and at the end of storage period in all treatments; this might be due to the severe of heat treatments of milk and the role of lactic acid bacteria in preservation of the products which associated with their ability to produce a range of antimicrobial compounds. These results are in agreement with those of Molan (1992). Furthermore,

Table 3. Influence of storage period on the chemical composition of yoghurt fortified with sedr honey

Storage days /Treatments	Titratable Acidity (%)				
	control	T1	T2	T3	Average
0	0.81	0.98	1.00	1.47	1.069^{D}
3	0.93	1.30	1.50	1.50	1.307^{C}
7	0.98	1.40	1.68	1.67	1.431^{B}
14	1.00	1.54	1.70	1.90	1.55^{A}
Average	0.945^{D}	1.31^{C}	1.47^{B}	1.633^{A}	
	Fat (%)				
0	3.90	3.97	3.99	4.00	3.965^{D}
3	3.92	3.98	4.03	4.13	4.015^{C}
7	3.94	3.99	4.06	4.23	4.055^{B}
14	3.96	4.00	4.13	4.25	4.085^{A}
Average	3.93^{D}	3.985^{C}	4.053^{B}	4.153^{A}	
	Total Protein (%)				
0	3.33	4.21	4.23	4.39	3.79^{A}
3	3.81	3.30	4.22	4.45	3.695^{A}
7	2.99	2.68	3.04	3.48	3.049^{B}
14	1.98	2.23	2.30	3.43	2.485^{C}
Average	2.777^{C}	2.856^{C}	3.447^{B}	3.937^{A}	
	Total Solids (%)				
0	13.30	13.66	13.96	15.76	14.173^{D}
3	14.12	14.06	15.23	17.53	15.239^{C}
7	15.48	15.58	16.11	18.87	16.51^{B}
14	15.96	16.51	16.88	19.58	17.237^{A}
Average	14.885^{C}	14.956^{C}	15.380^{B}	17.938^{A}	
	Ash (%)				
0	0.88	0.78	0.71	0.69	0.741^{A}
3	0.84	0.69	0.68	0.65	0.715^{A}
7	0.81	0.66	0.61	0.60	0.67^{B}
14	0.80	0.62	0.59	0.56	0.64^{B}
Average	0.83^{A}	0.664^{B}	0.647^{BC}	0.626^{C}	

Table 4. Influence of storage period on the bacteriological analysis of yoghurt fortified with sedr honey

Microbiological load (CFU $\times 10^6$/g)	Treatments	Storage days			
		0	**3**	**7**	**14**
Total bacterial	Control	110	177	180	183
	T_1	84	137	154	155
	T_2	83	120	138	139
	T_3	65	150	176	187
Str. thermophillus	Control	32	33	40	60
	T_1	26	31	38	52
	T_2	24	31	32	50
	T_3	20	30	35	48
Lb. bulgaricus	Control	55	44	46	68
	T_1	30	31	41	57
	T_2	15	30	31	44
	T_3	27	41	40	56

Yeasts & Moulds	Control	ND*	ND	31	31
	T_1	ND	ND	36	34
	T_2	ND	ND	35	33
	T_3	ND	ND	34	31

Table 5. Influence of storage on organoleptic properties of yoghurt fortified with sedr honey

Sensory Properties	Treatments	Storage days			
		0	3	7	14
Flavour (45)	Control	37	39	38	41
	T_1	38	40	42	43
	T_2	40	41	43	43
	T_3	36	38	39	40
Body and Texture (30)	Control	20	27	28	28
	T_1	23	25	25	26
	T_2	16	18	20	19
	T_3	15	17	17	18
Appearance (15)	Control	8	10	13	14
	T_1	10	11	13	12
	T_2	8	10	11	13
	T_3	10	11	12	12
Acidity (10)	Control	6	6	7	8
	T_1	5	5	7	8
	T_2	5	6	6	8
	T_3	5	6	7	7
Overall Scores (100)	Control	71	76	82	86
	T_1	76	81	87	89
	T_2	69	75	80	83
	T_3	66	72	75	77

Table 6. Influence of storage on chemical composition of bioyoghurt fortified with sedr honey

Storage days	Titratable Acidity (%)				
	Control	T1	T2	T3	Average
0	0.70	1.00	1.50	1.40	1.15d
3	0.90	1.60	1.70	1.67	1.466^{c}
7	0.99	1.63	1.73	1.80	1.515^{b}
14	1.00	1.80	1.87	1.93	1.650^{a}
Average	0.875^{c}	1.507^{b}	1.70^{a}	1.70^{a}	
	Fat (%)				
0	3.93	4.00	4.30	4.40	4.158^{d}
3	3.97	4.10	4.37	4.47	4.228^{c}
7	3.99	4.20	4.40	4.50	4.250^{b}
14	4.00	4.20	4.30	4.50	4.273^{a}
Average	3.973^{d}	4.125^{c}	4.342^{b}	4.468^{a}	
	Total Protein (%)				
0	4.07	5.14	5.67	4.50	4.845^{a}
3	4.06	4.66	5.22	4.39	4.588^{b}
7	3.09	4.00	5.00	3.67	3.940^{c}
14	2.68	3.46	3.91	2.86	3.258^{d}
Average	3.55^{d}	3.81^{c}	4.32^{b}	4.95a	
	Total Solids (%)				
0	11.00	12.43	12.88	16.97	13.32^{d}

3	13.48	14.21	14.74	16.99	14.855[c]
7	13.62	14.14	15.58	17.92	15.315[b]
14	14.16	14.68	15.97	17.97	15.695[a]
Average	13.065[d]	13.865[c]	14.792[b]	17.462[a]	
	Ash (%)				
0	0.78	0.74	0.72	0.68	0.730[a]
3	0.75	0.72	0.71	0.66	0.710[b]
7	0.69	0.67	0.60	0.59	0.637[c]
14	0.63	0.62	0.57	0.53	0.587[d]
Average	0.71[a]	0.69[b]	0.65[c]	0.62d	

Table 7. Influence of storage on bacteriological analysis of bioyoghurt fortified with sedr honey

Microbiological Properties (CFU $\times 10^6$/g)	**Treatments**	**Storage days**			
		0	**3**	**7**	**14**
Total bacterial	Control	70	140	160	164
	T_1	96	270	275	280
	T_2	187	210	250	290
	T_3	121	257	261	270
Str. thermophillus	Control	26	35	47	43
	T_1	20	54	51	60
	T_2	40	60	72	89
	T_3	26	64	66	70
Bifidobacteria	Control	27	38	33	35
	T_1	21	31	34	39
	T_2	40	41	44	48
	T_3	16	40	50	70
Yeasts & Moulds	Control	ND*	ND	37	35
	T_1	ND	ND	39	32
	T_2	ND	ND	47	30
	T_3	ND	ND	35	48

Table 8. Influence of storage on organoleptic properties of bioyoghurt fortified with Sedr honey

Sensory Properties	**Treatments**	**Storage days**			
		0	**3**	**7**	**14**
Flavor (45)	Control	28	29	30	33
	T_1	40	41	44	44
	T_2	35	36	37	38
	T_3	36	39	39	40
Body and Texture (30)	Control	20	25	28	29
	T_1	23	24	25	25
	T_2	20	22	22	23
	T_3	18	20	23	24
Appearance (15)	Control	11	12	13	14
	T_1	11	12	12	12
	T_2	8	10	10	10
	T_3	8	10	11	12
Acidity (10)	Control	6	7	8	9
	T_1	5	5	5	7
	T_2	5	5	6	5

	T_3	5	5	5	5
Overall Scores (100)	Control	65	73	79	85
	T_1	79	82	86	88
	T_2	68	73	75	78
	T_3	67	76	78	81

the aerobic and anaerobic bacteria counts were not detected in all treatment except 15% honey bioyoghurt at the 7 day to the end of storage period, this might be due to the microbial load of added honey and the post contamination after manufacture and at the end of storage period in all treatments; this might be due to the severe of heat treatments of milk and the role of lactic acid bacteria in preservation of the products which associated with their ability to produce a range of antimicrobial compounds. Nearly the same results are recorded by Rashid and Thakur (2012).

Organoleptic properties of bioyoghurt fortified with sedr honey

Bioyoghurt containing 5% of Sedr honey recorded the highest values for all sensory attributes as compared to other treatments at the end of storage period.

Vitamin content

Data in Table 9 shows that the vitamin content in these treatment were 0. 2358, 0.0366, 0. 4446 and 18.6499 for vitamins A, D, E and C respectively. These results are partly in agreement with ((Najgebauer-Lejko *et al*., 2014).

Table 9. Vitamins content (ppm) of the most favorite treatments at sensory properties

Vitamin (ppm)	A	C	D	E
Yoghurt with 5% sedr honey	0. 2358	18.6499	0.0366	0. 4446

Phenols Content

The concentrations of phenolic compounds in bioyoghurt fortified with 5% sedr honey (Day14) are given in Table 10. From this table it could be observed that the amount of Gallic, Pyrogallol , Amino benzoic, Proto Catchoic, Chlorogenic, Epicatechin, Caffeine, Caffeic, P.Coumaric, Benzoic, 3,4,5methoxy cinnamic were detected. These results are lower than those of (Cossu *et al*., 2009).

Table 10. Some phenols content (ppm) of the most favorite treatments of sensory properties

Phenolic compounds	Amount in ppm
Gallic	0.0596
Pyrogallol	2.3519
Amino benzoic	0.05011
Proto Catchoic	0.3141
Chlorogenic	1.5665
Epicatechin	0.1749
Caffeine	0.2969
Caffeic	0.0700
P.Coumaric	0.0883
Benzoic	0.5675
3,4,5methoxy cinnamic	0.0381

CONCLUSION

Addition of honey increased the total solids content of the product thereby raise the nutrition value. Bifidobacteria were greatly activated by mixing of honey with yoghurt milk which mean that honey could be utilized as sweeter and prebiotic in bioyoghurt production. The result of the organoleptic properties of yoghurt and bioyoghurt cleared that there was difference in color, appearance, body, texture and flavor. Incorporation of honey highly improved the sensory evaluation scores of the resulted yoghurt. Finally, this study recommends that it can use probiotic bacteria and herbs honey to make good functional dairy foods.

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Conflict of interest

The authors declare no conflict of interest.

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Biological Evaluation of Fermented Camel Milk Drinkable like Soup Fortified with Different Cereals

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ABSTRACT

Diabetes mellitus (DM) is caused by an imbalance in metabolism of carbohydrates and lipids regulated by insulin. This work aimed to assess the therapeutic efficiency of diets contained fermented camel milk soup prepared using some different cereals. Alloxan (intraperitoneal injection of 150mg/ kg body weight) was applied for inducing diabetes and hyperlipidemia. Five groups, each of six rats, including negative and positive (diabetic rats) controls were fed on basal diet. In addition to basal diet, diabetic rats in group A, B and C were fed on fermented camel milk wheat soup, fermented camel milk barley soup, and fermented camel milk oat soup, respectively. After six weeks of feeding, results showed significant decrease ($P < 0.05$) in levels of glucose comparing with diabetic rats. However, significant decrease in the levels of SGPT, SGOT, TC, TG, LDL-c, VLDL-c and AI in treated rats were observed. The present study indicated that, diets contain fermented camel milk soup prepared using different cereals could be providing several advantages in treatment and management of diabetes in addition to reduce the risk of diabetic complications.

Keywords: Cereals. Camel milk. Diabetic rats. Lipid Profile. Liver enzymes.

INTRODUCTION

Diabetes mellitus (DM), is an epidemic disease prevalenced around the world. The global prevalence of the disease is expected to increase from 2.8% in 2000 to 4.4% in 2030 (Shehata and Moussa, 2014). That increase manly has been predicted occur in the developing countries. *Diabetes mellitus* is caused by an imbalance in metabolism of carbohydrates and lipids regulated by insulin (Radhika *et al*., 2011). High levels of blood glucose or hyperglycemia is a direct result to *diabetes mellitus* disease. The chronic hyperglycemia led to failure of organs. Hyperlipidemia as a result to lipolysis in adipose tissue is accompanying to *diabetes mellitus* disease (Goodman *et al*., 2006).

Camel's milk could be differentiated from other milks in some characteristics such as low in cholesterol, lactose, free from β- lactoglobulins, high in some minerals, some vitamins and contains high concentration of insulin (Agrawal *et al*., 2011). Camel milk characterized by contains polyunsaturated and hydroxy fatty acids. High content of lysozyme, lactoferrin and immunoglobulins give the camel milk a good biological value. Camel milk is known to have some medicinal properties since ancient times (Agrawal *et al*., 2007a).

Given the importance of fermented camel milk health and nutritional value, studies have focused on the raising of the nutritional value through consolidation of grain. El-Gendy *et al*. (2016) have manufactured fermented camel milk fortified with some cereals such as barley, oats and wheat to prepare different Kishk powder preparations.

Thus, this work was done to study the therapeutic efficacy of fermented camel milk product fortified with some cereals in the feeding of diabetic male albino rats.

MATERIAL AND METHODS

Experimental animals

The experiment was conducted on 30 male albino rats with an average weight of (180 ± 10g) were obtained from the Organization of Biological Products and Vaccines (Helwan Farm, Cairo, Egypt).

The rats were housed in screen-bottomed aluminum cages in rooms maintained at 25±1°C with alternating cycles of light and dark of 12hr duration. The control diet was used to feed the rats for seven consecutive days. Six rats were randomly chosen (initial group), blood samples were withdrawn from the retro-orbital plexus of the eyes from each rat according to the procedure of (Schermer, 1967). Serum was separated and the serum biochemical analyses were estimated. Rats were divided into five groups, each group contains six rats (Table 1).

Basal Diet

The basal diet consists of casein (14%), corn oil (10%), choline chloride (0.25%), vitamin mixture (1%), cellulose (5%), mineral mixture (4%), Dl-methioinine (0.3%) and corn starch (up to 100%), (Reeves *et al*., 1993).

Soup prepared

Kishk powder (fermented camel milk soup prepared using different cereals such as wheat, oat, and barley has been produced in the previous work of our research group (El-Gendy *et al*., 2016). To prepare Kishk soups, 20 g of each Kishk powder was cooked in 200 ml water. All samples were boiled for 10 min over medium heat with constant stirring.

Table 1. Animal groups used in the present study

Groups	Description
NC	Rats fed on basal diet.
PC	Diabetic rats fed on basal diet.
A	Diabetic rats fed on basal diet with fermented camel milk wheat soup.
B	Diabetic rats fed on basal diet with fermented camel milk barley soup.
C	Diabetic rats fed on basal diet with fermented camel milk oat soup

NC= Negative control.

PC= Positive control

Induction of diabetes

The animals were fast overnight, and received a single intraperitoneal injection of freshly prepared alloxan using citrate buffer 0.1M (pH = 4.5) as vehicle, at a dose of 150 mg alloxan/kg body weight (Szkudelski, 2001). Rats in the negative control group were injected with citrate buffer alone. On the third day of alloxan injection, blood glucose was determined and animals with glucose level more than 200 mg/dl were considered as diabetic rats.Soups were given orally to the rats daily in a dose of 1 ml/100 g body weight for successive 6 weeks. Calculated dose was based on a consumption of 275 ml/day for a 70 kg human as reported by (Rouanet *et al*., 2010).

Biochemical Analyses

Serum biochemical Analyses

Blood glucose level was estimated as described by Trinder (1969). Fully enzymatic determination of total triglycerides in serum was estimated by the method described by Fossati and Prencipe (1982). Clolesterol was assayed as mentioned by Roeschlau *et al*., (1974). Low density lipoproteins cholesterol was determined according to Wieland and Seidel (1983). (LDL-c) were precipitated by heparin at (pH 5.04). After centrifugation, the remained (HDL-c) and (VLDL-c) were estimated in the supernatant. LDL-c = Total cholesterol - cholesterol in the supernatant. The high density lipoproteins-cholesterol (HDL-c) was determined according to the method of Lopez-Virella *et al*., (1977). Atherogenic index of serum (AI) was estimated according to Dobiášová and Frohlich (2001).

Determination of liver enzymes

The colorimetric determination of Aspartate aminotransferase (AST/GOT) and Alanine aminotransferase (ALT/GPT) were carried out according to the method of Sherwin (1984).

Statistical analyses procedure

Data for biological study were expressed according to analyses of variance for repeated measurements. The analyses were conducted with SAS program (SAS, 2004).

RESULTS AND DISCUSSION

The effect of feeding fermented camel milk soup prepared using some different cereals on levels of glucose in diabetic rats were shown in Figure 1. Data in illustrated that a significant increase (P <0.05) in level of glucose (361.33 mg/dl) of diabetic group (positive control group), which treated with a single dose of alloxan, compared to negative control group (93.26 mg/dl). Similar results have been obtained by Akpan, 1989 and Radhika *et al*., 2011. They demonstrated that, alloxan administration was associated with hyperglycemia. Hyperglycemia due to produce a reproducible model of *diabetes mellitus*. that had minimal beta cell activity. After six weeks of administration of fermented camel milk wheat soup, barley soup and oat soup reduce level of glucose by 145.60, 95.53 and 78.67 mg/dl, respectively. Furthermore, the highest reduction in glucose level was in group (C) diabetic rate fed with fermented camel milk oat soup

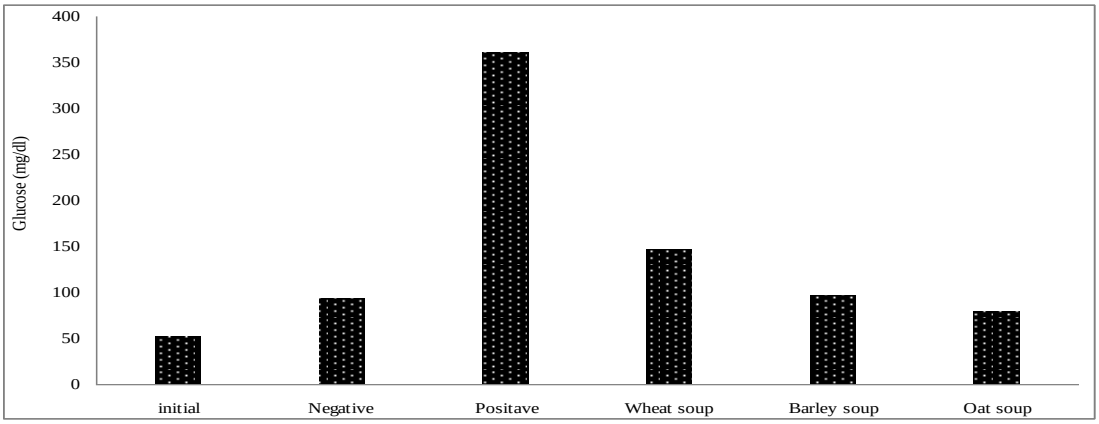

Fig. 1. Effects of diets containing fermented camel milk soup prepared using cereals on serum glucose level

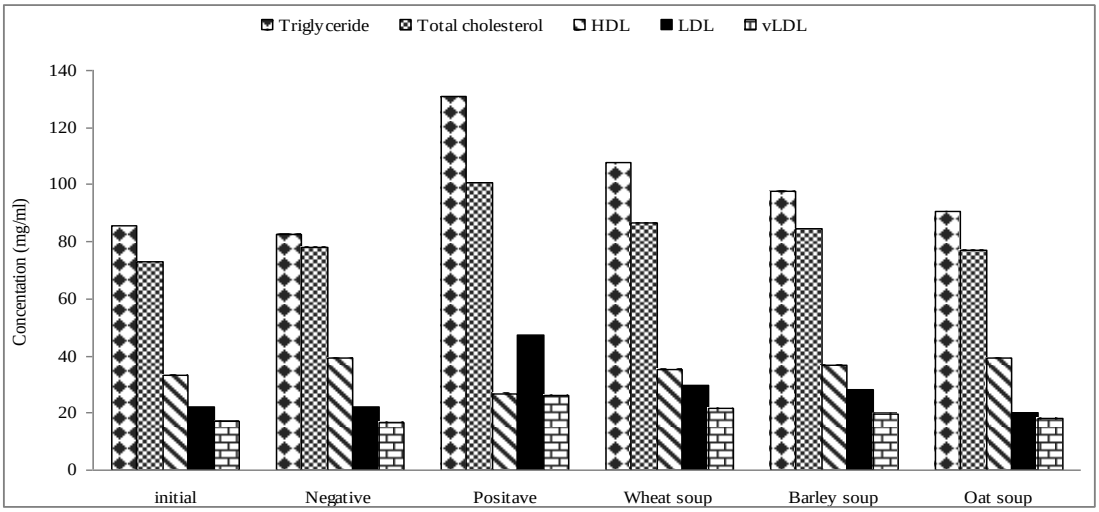

Fig. 2.Effect of diets containing fermented camel milk soup prepared using cereals on lipid profile

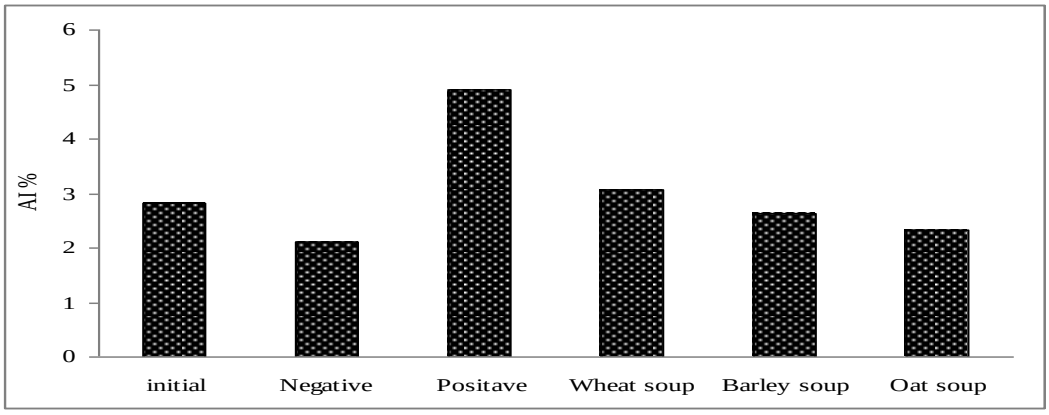

Fig. 3. Effect of diets contain fermented camel milk soup prepared using cereals on atherogenic index

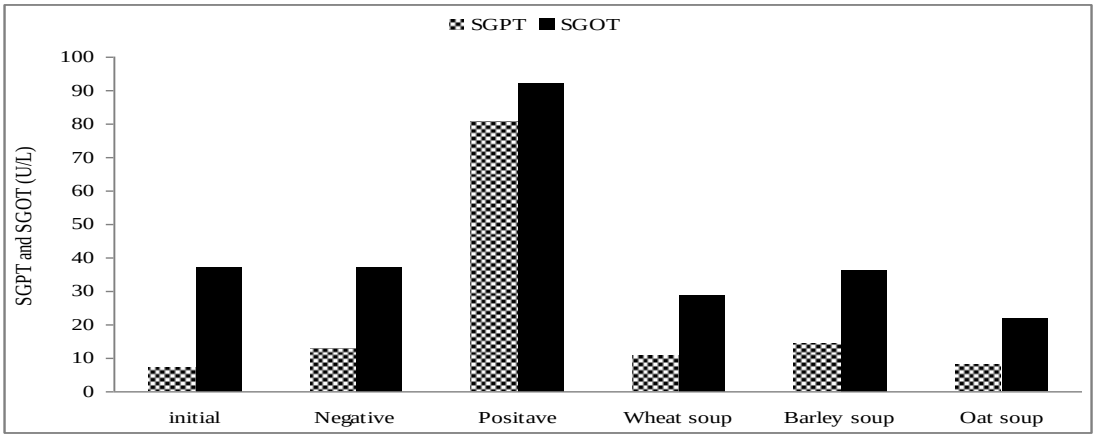

Fig. 4. Effect of diets contain fermented camel milk soup prepared using cereals on liver enzymes

Also, the obtained results demonstrated the therapeutic activity of fermented camel milk oat soup. Advantageous control in blood sugar have been reported due to uptake the soluble fiber β-glucans (from oats) by type 2 diabetic patients (Jenkins *et al*., 2002 and Tapola *et al*., 2005). Moreover, little amounts of β-glucans (from oats) have been suggested to moderate both glucose and insulin response after carbohydrate rich meals (Hallfrisch *et al*., 1995, Alminger and Eklund-Jonsson 2008 and Tosh, 2013). In general, β-glucans could be suggested to alter insulin delaying or preventing type 2 diabetes (Pereira *et al*., 2002, Steffen *et al*., 2003, Angela *et al*., 2003 and Maki *et al*., 2007). The obtained results are in consistent with those mentioned in several previous studies (Jenkins *et al*., 1988, Liljeberg *et al*., 1992 and Granfeldt *et al*., 1995). These studies indicated that, the boiled whole oats caused little response in glucose level and insulin, however this effect have been improved when oats were grounded to flour before cooking.

In accordance with the results obtained, following oats, barley showed ability to reduce blood sugar levels. This is consistent with Choi *et al*., (2010), who stated that barley nutrition can enhance blood sugar level management. Therefore, barley could be considered as a smart grain choice to slow down the releasing of sugar into the blood stream. This effect may be due to high β-glucans content of barley (Rendell *et al*., 2005).In addition, the use of camel milk in controlling the level of insulin and sugar in the blood. This is consistent with Ejtahed *et al*. (2015) when using camel milk to patients with type 2 diabetes mellitus and might incorporate in glycemic control. Also Beg *et al*. (1986) and Farah (1993) found the containment of camel milk to some amino acids such as half- cystine and a number of minerals and vitamins, which play a role as anti-oxidant thereby removing free radicals, which all contribute to the impact on blood sugar.

The results in figure (2) showed that a significant increase P <0.05 in total cholesterol (TC), total triglycerides (TG), low density lipoprotein (LDL-c) and very low density lipoprotein (VLDL-c) of diabetic group compared to Negative control group. While, HDL-c decreased significantly P <0.05 as compare to Negative control group. A significant increase in TG (PC) may be due to the lack of insulin under diabetic condition. The obtained result closed with that of (Arkkila *et al*., 2001) who found that the disturbance in the lipid metabolism may be due to insulin deficiency.

While, the groups fed with fermented camel milk wheat soup, fermented camel milk barley soup and fermented camel milk oat soup decreased significantly p < 0.05 the levels of TC, TG, LDL-c and VLDL-c as compared with positive control group and this was associated with a significant increase p < 0.05 in HDL-c in these groups.

TG in fermented camel milk oat soup (90.80 mg/dl) was significantly lowest p <0.05 as compare with diabetic group. These results were in accordance with those mentioned by Hull, 2004 and Agrawal *et al*., 2007b. They stated that the high insulin content of camel milk can cause activation of lipoprotein lipase enzyme. Furthermore, there are decreases in total cholesterol; rate of decrease was 86.38, 84.43 and 77.28 mg/dl, respectively of fermented camel milk wheat soup, fermented camel milk barley soup and fermented camel milk oat soup as compared to the diabetic group (Positive control). In general, we can say that the best effect as absolute value was for fermented camel milk oat soup followed by fermented camel milk barley soup then fermented

camel milk wheat soup with non-significant small differences in lipid profile values.

Harris and Crabb (1982) have pointed out that there is a relationship between diabetes mellitus and hypercholesterolemia, hyperlipidemia and hepatic steatosis. The hypercholesterolemia occurred as a result of availability of aceyl CoA in high amount, due to accelerated fatty acid oxidation, followed by increasing cholesterol synthesis (West *et al*., 1983). Similarly, the hyperlipidemia associated with diabetes mellitus due to release of VLDL-c without equivalent increase in the level of degradation by the lipoprotein lipase whose activity is dependent on high insulin: glucagon ratio.

Several studies have demonstrated lowering effect of oats on cholesterol level in blood due to its high content of β-glucan (Agot *et al*., 2003, Chen *et al*., 2006 and Thies *et al*., 2014). It is also evident that the beta-glucans have the ability to lower cholesterol either through reduce the absorption of fats and cholesterol (Wang *et al*., 1992). Also, β-glucan may bind with cholesterol-rich bile acids in the intestine, and carry them out of the body (Kahlon *et al*., 1993). Moreover, Behall *et al*. (2004a) stated that, the consumption of barley, which is rich in beta-glucan and insoluble fiber has, contributed to reducing a lower incidence of heart disease.

The effect of tested diets contain fermented camel milk soup prepared using some different cereals on atherogenic index for 6 weeks on serum atherogenic index (AI) in diabetic rats is showed in figure 3. The mean value of (AI) in positive control is highest that of all tested diets. In the other hand an elevation in AI on the groups fed with fermented camel milk wheat soup, fermented camel milk barley soup and fermented camel milk oat soup compared to negative control and initial group were showed non-significant($P<0.05$). Similarly, Behall *et al*. (2004b) has explained many cardiovascular risk factors can be reduced due to eating barley rich in soluble fiber.

A comparison of the liver enzymes parameters data for the fermented camel milk wheat soup, fermented camel milk barley soup and fermented camel milk oat soup administration is shown in figure 4. A significant increase $p < 0.05$ in the levels of liver enzymes (SGPT and SGOT) appeared in diabetic rats these results are accordance with data reported by Kumar *et al*., 2011 who indicated that the liver enzymes SGPT and SGOT levels were increased in alloxan diabetic rats. This elevation may be due to release of SGPT and SGOT from damaged hepatic cells. Significant ($p < 0.05$) enhancement in liver functions parameters was obtained by diabetic rat groups of fermented camel milk oat soup followed by fermented camel milk wheat soup and fermented camel milk barley soup. In accordance with our results, Magjeed, (2005) and Khan and Alzohairy, (2011) mentioned that giving camel milk improved the levels of SGPT and SGOT activities in intoxicated rats.

CONCLUSION

This study has demonstrated the therapeutic efficiency of feeding fermented camel milk soup prepared using different cereals for diabetic rats. Also, results indicated that consuming fermented camel milk soup prepared using different cereals have anti-diabetic potential in addition to hepato protective and hypolipidemic effects in alloxan-induced diabetic rats. In general, fermented camel milk soups prepared in the present study may have significant applications for the clinical management of *diabetes mellitus* in humans.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Isolation and Identification of coagulase positive *Staphyllococcus Aureus* in dairy products marketed in Chittagong, Bangladesh and analysis of antimicrobial susceptibility profiles

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ABSTRACT

The present study was performed to verify the presence of *Staphylococcus aureus* in dairy products marketed in Chittagong city. 100 samples of different dairy products such as Cheese (20), Ice-cream (20), Butter (20), Sweetmeats (20), Yoghurt (20) were collected and analyzed. After isolation of *S. aureus* on MSA the 27 positive isolates were further subjected to coagulase test. Among them 19 isolates were found positive in both the tube and slide coagulase tests. Then 19 coagulase positive *S. aureus* isolates were subjected to antimicrobial resistance profile assessment for phenotypic investigation of MRSA. All the 19 coagulase positive *S. aureus* isolates were resistant to Oxacillin showing no zone of inhibition but, in case of Cefoxitin only 10 isolates were found resistant to it where the zone of inhibition was below 21 mm and other 7 isolates showing intermediate sensitivity and 2 isolates showing full sensitivity to Cefoxitin. Conversely 8 isolates were resistant to gentamycin where the inhibitory zone was ≤12 mm and 10 and 1 were found as intermediate and sensitive to it respectively. Use of ciprofloxacin and kanamycin discs for differential diagnosis of MRSA revealed that out of 19 coagulase positive isolates of *S. aureus* 17 showed sensitivity where the inhibitory zone was ≥31 mm and 2 showed intermediate results where the inhibitory zone was between 22-30 mm. The results demonstrate presence of high level of coagulase positive *S. aureus* in dairy products that causes potential public health risks. Proper pasteurization of milk can limit the spread of MRSA to human body.

KEY WORDS: Dairy products, Public health, Antimicrobial Susceptibility, Pasteurization MRSA

INTRODUCTION

Milk contains about 100,000 different molecular species in different states of dissemination. Milk is called an ideal nutrition source for microorganisms and has a pH value that is needed to contribute to proliferation and thus is an excellent media for the growth and multiplication of *S. aureus* also (Asperger *et al.*, 2003). The most important mammary gland pathogen S. aureus is responsible for bovine mastitis. It causes extent amount of economic losses to dairy farmers (Hata *et al.*, 2008). It contaminates milk either by direct excretion from udders or by secondary contamination or due to processing, handling and unhygienic environment (Scherrer *et al.*, 2004). Furthermore, *S. aureus* also causes various human diseases such as impetigo, abscesses, endocarditis, toxic shock syndrome, food borne intoxication, and staphylococcal scalded skin syndrome (Ladhani *et al.*, 1999). S. aureus also causes zoonotic disease that can be spread between humans and cows (Matos *et al.*, 1991). Heating at normal cooking temperature can killed the bacterium but the toxins remains active (Presscott *et al.*, 2002). Staphylococcal enterotoxins are categorized to be more heat resistant in foodstuffs than in a laboratory culture medium because they are highly heat resistant (Bergdoll, 1983).

Staphylococcus isolates is usually divided into coagulase positive and negative. The coagulase positive isolates are regarded as more virulent and this group includes the species of *S. aureus, S. hyicus* and *S. intermedius* (Quinn *et al.*, 1999). Coagulase is an enzyme that converts fibrinogen to fibrin. In the laboratory, this characteristic is used to distinguish between different species of Staphylococcus. Free and bound coagulase coats the bacterial cells with fibrin, which shield them from opsonization and phagocytosis. Combination of toxin-mediated virulence, invasiveness and antibiotic resistance makes S. aureus as the most important coagulase positive pathogen from staphylococci groups (LeLoir *et al.*, 2003).

Antibiotic resistant strain of *S. aureus has been treated as a raising concern in recent years*. The extensive therapeutic use of antimicrobials and used as growth promoters in food animal production

develop create more resistance (Normanno *et al*., 2007). Isolates of *S. aureus* are habitually resistant to methicillin (oxacillin) and rest of all β-lactam antibiotics. An organism with this type of resistance is referred to as methicillin-resistant *S. aureus* or MRSA (Lee, 2003). MRSA infections are more difficult to treat with standard antibiotics and thus more dangerous.

The concern about the public health impact of MRSA associated with food producing animals is increased at now-a-days. MRSA along with their their resistance genes can spread from animals to humans by direct contact or through the food chain (Kluytmans, 2010). MRSA strains have been isolated in many countries from cows' or small ruminants' milk and various dairy products (Vyletelova *et al*., 2001, Juhasz-Kaszanyitzky *et al*., 2007; Normanno *et al*., 2007; Turutoglu *et al*., 2006, Ateba *et al*., 2010; Hata *et al*., 2010). Considering the potential of the transmission of MRSA from cows to humans, the present study was under taken with listed below objectives: to determine the level of *S. aureus* contamination in dairy products marketed in Chittagong, to isolate and identify the coagulase positive staphylococci in dairy products and to analyze the antimicrobial susceptibility profiles of coagulase positive staphylococci for phenotypic investigation of MRSA.

MATERIALS AND METHODS

Sample collection

Different dairy products available in Chittagong city such as, Cheese (20), Ice-cream (20), Butter (20), Sweetmeats (20), and Yoghurt (20) were collected aseptically and transported in an ice-box to laboratory and kept 0ºC until investigation.

Bacteriological Investigation

For enrichment of each sample Peptone Water (PW) enrichment broths were used (HiMedia Pvt. Ltd.). 10 g of each sample was homogenized with 90 ml sterile enrichment broth peptone water and enriched for 24 hrs at 37°C.

Initial screening of *S. aureus* was done from the collected samples by inoculum a loopful of each enrichment were streaked onto Mannitol salt agar medium (Oxoid, Basingstoke, Hampshire, UK). *S. aureus* produced bright yellow coloured colonies after incubation of 24 hrs at 37ºC in these medium. Mannitol salt agar medium was prepared according to the instructions of manufacturer (Oxoid, England). The positive colonies with bright yellow zones (due to mannitol salt fermentation) were then re-inoculated into blood agar to detect the pathogenecity of *S. aureus*. All streaked blood agar plates were incubated at 37ºC overnight to allow hemolysis to occur. After that five such cross-sectional colonies were picked up and transferred to a 10 ml test tube containing 5 ml of tryptic soy broth (TSB)that were prepared according to the instructions of manufacturer (Oxoid, England), incubated at 37°C for 6 h. The broth cultures were then considered for the coagulase test.

Morphological characteristics

The smear from the isolated culture was constructed on clean grease free microscopic glass slide. Then stain with Gram's Method of staining. The smear was observed under microscope. Smear divulge Gram positive, spherical cells arranged in irregular clusters resembling to bunch of grapes.

Coagulase test

To conduct the coagulase test, whole blood from horse was collected into commercially available EDTA –treated lavender tops. Then blood was centrifuged at 2600 rpm for 10 minutes using a refrigerated centrifuge. The resulting supernatant, the plasma was then immediately transferred to a sterile 1.5 ml eppendorf tube using a sterile micropipette. The plasma was stored at -20ºC for future use.

Tube coagulase test

Sterile tubes containing 50 µL of horse plasma were collected. From each tube cultivation in TSB 50 µL was transferred to sterile tubes. The incubation was done at a temperature of 35ºC±2ºC for 6 hours. The presence of coagulates were verified, taking into consideration the following criteria:

Reaction 1+: Small disorganized coagulation.

Reaction 2+: Small organized coagulation.

Reaction 3+: Large organized coagulation.

Reaction 4+: Coagulation of all the contents of the tube which do not come off when inverted.

When coagulation reaction type 3+ and 4+ occurred, the presence of Staphylococcus aureus was confirmed.

Slide coagulase test

For confirmation of *S. aureus* some selected isolates were subjected to slide coagulase test. One drop of the horse plasma was placed on clean glass slides that are grease free. A small loop of the *S. aureus* suspected culture was mixed with plasma separately and checked for agglutination. The cultures showing

agglutination were recorded as positive for coagulase test and thus confirmed as *S. aureus*.

Microbial resistance testing of coagulase positive *S. aureus*

A disk diffusion method on Mueller-Hinton agar (Oxoid) was used to evaluate phenotypic methicillin (oxacillin) resistance according to the Clinical Laboratory Standards Institute standards. Muller-Hinton (MH) agar containing 2% NaCl was prepared according to the Bauer-Kirby disk-diffusion procedure (Bauer et al., 1966). A bacterial turbidity equivalent of 0.5 Mcfarland standards was used as inoculum for each isolate. 0.5ml of 1% (11.75 g/L) $BaCl_2.2H_2O$ was added to 99.5ml of 1% (0.36N) H_2SO_4 (Carter and Cole, 1990) for the preparation of Mcfarland standard. The antibiotic resistance pattern for the panel of antibiotics was determined considering the zone of inhibition sizes for each of the antibiotics as "resistant (R)", "intermediately resistant (I)", and "sensitive (S)" against the test isolates as recommended by the Clinical and Laboratory Standard Institute (CLSI, 2007). A sterile swab was dipped into the inoculums, prepared for antimicrobial sensitivity test, and rotated against the side of the tube with firm pressure. Then after removing the excess fluid from the swab the dried surface of MH agar was inoculated by streaking the swab three times over the entire agar surface rotating the plates approximately at 60 degrees for each time to ensure an even distribution of the inoculums. The antimicrobial disks were placed on the surface of the inoculated agar. Each of the antimicrobial disks was used to dispense separate forceps. The disks were inserted carefully on the surface of the agar with a gentle pressure to make a complete contact. After dispensing all of the disks the agar plate was incubated at 35°C for 16 to 18 hours. At the end of incubation the size of zone of inhibition around a micro-disk was measured with a digital slide calipers and the result was deduced according to CLSI, 2007.

RESULTS AND DISCUSSION

Antimicrobial resistance pattern of coagulase positive *S. aureus*

After performing coagulase tests all the 19 coagulase positive *S. aureus* isolates were subjected to antimicrobial resistance profile assessment for phenotypic investigation of MRSA. MRSA is indicated by assessing zone of inhibitions with oxacillin $\leq$ 14 mm and/or cefoxitin $\leq$ 21 mm (CLSI, 2007). For this reason two types of discs containing Oxacillin or Methicillin (1µg) and Cefoxitin (30µg) were used for confirmation of MRSA. The other three discs containing Ciprofloxacin (5µg), Kanamycin (10µg) and Gentamycin (10µg) were used mainly for differential diagnosis of MRSA. The results in Table (2, 3) showed the antimicrobial resistance pattern of coagulase positive *S. aureus*. It is evident from the table that all the 19 coagulase positive S. aureus isolates were resistant to oxacillin showing no zone of inhibition but, in case of cefoxitin only 10 isolates were found resistant to it where the zone of inhibition was below 21 mm and

Coagulase assessment

The present study was conducted for isolation and identification of coagulase positive *S. aureus* from the dairy products marketed in Chittagong. A total of 100 samples of different dairy products were collected between January 2015 and March 2015. In the current study, investigation of the level of coagulase positive *S. aureus* in different dairy products was done and then all the positive isolates were subjected to antimicrobial analysis for the confirmation of MRSA. After isolation of *S. aureus* on MSA the 27 positive isolates illustrated in Figure 1 were further subjected to coagulase test. Among them 19 isolates were found positive in both the tube and slide coagulase tests that shown in Table 1. In the slide coagulase tests, cultures which showed agglutination were recorded as positive for coagulase test outlined in Figure 2 and in tube coagulase examination, cultures which showed heavy coagulation of all the contents of the tube and that did not come off even after inverting the tube upside down were recorded as positive for coagulase test were shown in Figure 3.

It is well known that pasteurization of milk eliminates *S. aureus*. Raw milk and raw-milk cheese contaminated with MRSA have been incriminated in the transmission of the pathogen to humans (Normanno *et al*., 2007). Handling or consumption of raw milk and dairy products may lead to the spread of antimicrobial resistance genes of *S. aureus* to humans. On the basis of bacteriological culture 19 out of 100 (19.0%) samples were confirmed tentatively to be contaminated with the coagulase positive *S. aureus*. This finding closely related to the findings of Santana *et al*. (2010) found 18.8% of positivity in 101 samples from Londrina and Pelotas. But variation may arise in isolation of coagulase positive *S. aureus* that is attributable to geographic variation of the region from where the samples were collected, variation of the techniques adopted by different laboratories for conducting the experiments.

Table 1. Total number of coagulase positive isolates

Sample (Dairy products collected from Chittagong city)	*S. aureus* positive isolates	Coagulase test positive isolates
Cheese (n=20)	3	2
Ice-cream (n=20)	2	2
Butter (n=20)	5	3
Sweetmeats (n=20)	8	6
Yoghurt (n=20)	9	6
Total	27	19

Table 2. Antibiotic resistance pattern of coagulase positive *S. aureus* isolates

Antibiotic disks		OX (zone of inhibition: ≤ 14 mm), Antibiotics used:1µg			FOX (zone of inhibition: ≤ 21 mm), 30µg		
Resistance pattern		R(≤14 mm)	I (18-24mm)	S (≥25 mm)	R(≤21 mm)	I (22-30mm)	S(≥31 mm)
Coag. Positive staphylococci N=19	Cheese (n=2)	2	0	0	1	1	0
	Ice-cream (n=2)	2	0	0	0	1	1
	Butter (n=3)	3	0	0	2	1	0
	Sweetmeats (n=6)	6	0	0	3	2	1
	Yoghurt (n=6)	6	0	0	4	2	0
Total		19	0	0	10	7	2

R =Resistant, I =Intermediate, S =Sensitive, OX =Oxacillin, FOX =Cefoxitin

Table 3. Antibiotic resistance pattern of coagulase positive *S. aureus* isolates

Antibiotic disks		GEN (zone of inhibition: ≤ 12mm), 10 µg			CIP (5 µg) & K (10 µg)		
Resistance pattern		R(≤12 mm)	I (13-14)	S (≥15 mm)	R(≤21 mm)	I (22-30mm)	S (≥31 mm)
Coagulative Positive staphylococci N=19	Cheese (n=2)	0	2	0	0	0	2
	Ice-cream (n=2)	0	2	0	0	0	2
	Butter (n=3)	1	2	0	0	0	3
	Sweetmeats (n=6)	4	2	0	0	1	5
	Yoghurt (n=6)	3	2	1	0	1	5
Total		8	10	1	0	2	17

GEN=Gentamycin, CIP =Ciprofloxacin, K=Kanamycin

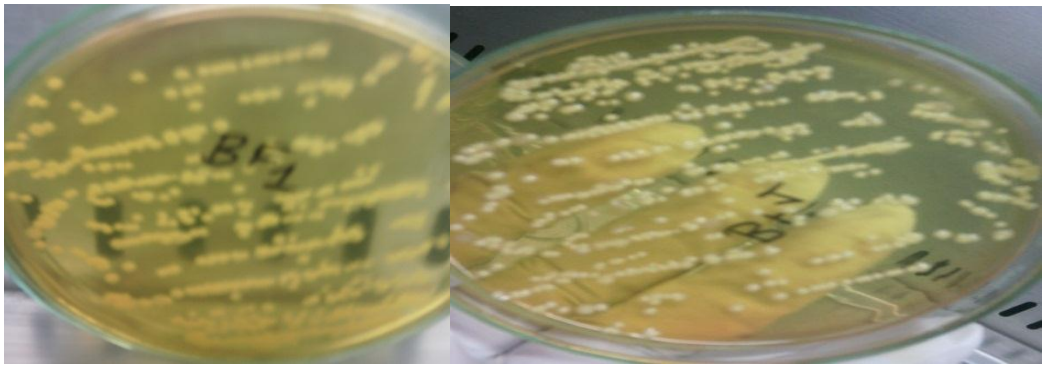

Fig. 1. Typical bright yellow colonies indicating the growth of *S. aureus* on Mannitol salt agar plates

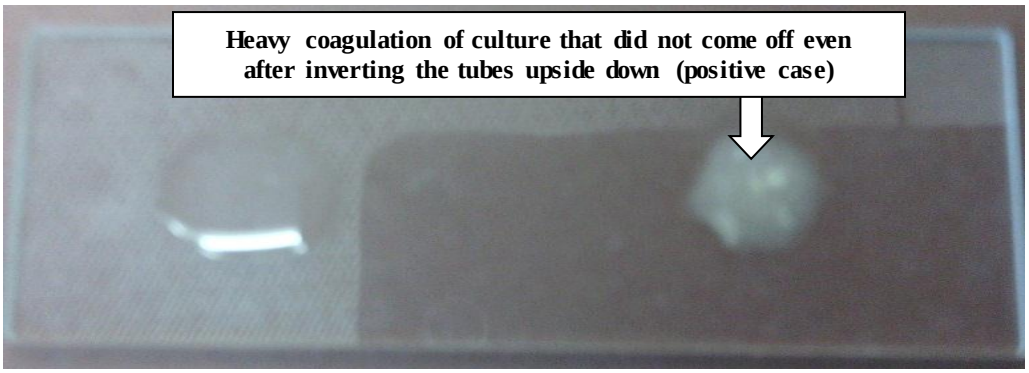

Fig. 2. Slide coagulase test for confirmation of *Staphyllococcus aureus*

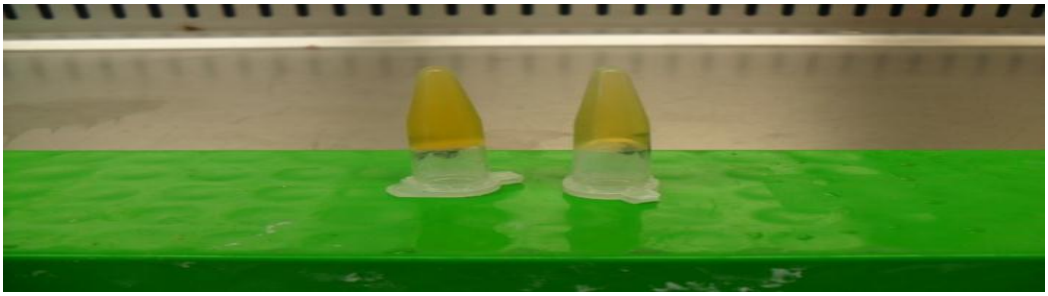

Fig 3. Slide coagulase test for confirmation of S*taphyllococcus aureu*

other 7 isolates showing intermediate sensitivity and 2 isolates showing full sensitivity to cefoxitin. On the other hand 8 isolates were found resistant to gentamycin where the inhibitory zone was ≤12 mm and 10 and 1 were found as intermediate and sensitive to it respectively. Use of ciprofloxacin and kanamycin discs for differential diagnosis of MRSA revealed that out of 19 coagulase positive isolates of *S. aureus* 17 showed sensitivity where the inhibitory zone was ≥31 mm and 2 showed intermediate results where the inhibitory zone was between 22-30 mm.

To determine whether the *S. aureus* isolates were methicillin resistant or not culture sensitivity (CS) test was done. For this we used Bauer-Kirby disk-diffusion procedure *(*Bauer *et al., 1966)* where disc diffusion test was performed by incubating *S. aureus* on Muller Hilton agar (MHA) impregnated with Oxacillin or Methicillin (1 or 5µg) and Cefoxitin (30µg) discs. Assessing zone of inhibitions with Oxacillin ≤ 14 mm and/or Cefoxitin ≤ 21 mm identified MRSA (CLSI, 2007). In our study it was found that all 19 test isolates were 100% resistant to

Oxacillin , 52.63% resistant to Cefoxitin and 42.11% resistant to gentamycin but most of the (89.47%) coagulase positive isolates sensitive to ciprofloxacin (CIP) and kanamycin (K). The probable reason between these variations may be, in case of Oxacillin false resistance is usually encountered due to hyper-production of ß-lactamase (Felten *et al*., 2002). Easy reading, higher sensitivity and results in comply with PCR (Broekema *et al*., 2009; Rao *et al*., 2011) makes Cefoxitin disc diffusion test more superior than Oxacillin disc diffusion test. So that accurate zone measurement is required and large zone sizes are produced by sensitive strains that could potentially interfere with other zones if multiple discs are tested on the same plate (Skov *et al*., 2005). These two reasons might have influenced our test resulting in showing low resistance in case of Cefoxitin. Kirkan *et al*. (2005) reported coagulase positive *S. aureus* from milk samples to be resistant to penicillin/oxacillin ranging from 87.5% to 95% correlates almost with these findings.

CONCLUSION

About 19% of the dairy products marketed in Chittagong were found to contain coagulase positive *S. aureus* the indicator bacteria for methicillin resistance. Phenotypically all the isolates showed methicillin resistance i.e., oxacillin resistance (100%) and cefoxitin resistance (52.63%). Presence of very high level of coagulase positive *S. aureus* showed that they were phenotypically determined to be methicillin resistant, in the dairy products indicating some public health risks. Therefore, to limit the spread of MRSA to humans the study recommends proper pasteurization of milk before preparing of any types of dairy products and have to ensure its proper hygienic practices during processing of milk for making modified milk products and proper handling and transportation until consumption.

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Optimization of α-amylase and amyloglucosidase productions from some amylolytic yeast strains

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ABSTRACT

The effect of certain environmental and nutritional factors on α-amylase and amyloglucosidase (AMG) production by highly amylolytic yeast isolates, *Cryptococcus heimaeyensis* HA7, *Candida membranifaciens* HA19, *Candida tropicalis* HA147 and *Candida sake* HA124 was investigated. The results concluded that there were variations among the tested yeast strains in amylase enzymes production affected by environmental and nutritional factors. The optimum pH value of α-amylase and AMG productions was at pH 6 for *Cry. heimaeyensis* and *C. sake* and pH 4.5 for *C. membranifaciens* and *C. tropicalis*. In addition, optimum incubation period was 48 h for all yeast strains except C. heimaeyensis which needed 72h. On the other hand, a marked similarity in the optimal temperature and the inoculum size for all yeast strains under study were observed (25°C and 1%, respectively). The trial experiments to obtain the highest yield of amylase enzyme productions resulted in modification of the recommended basal inorganic salts-starch medium. The highest yield of amylase production was obtained by increasing the soluble starch a sole source of carbon from 0.5 to 4% concentration for *C. tropicalis*, *C. membranifaciens* and *Cry. heimaeyensis while it was 5% for C. sake. The optimum levels of yeast extract was 1% for C. sake* and *C. membranifaciens*, while 2% for *Cry. heimaeyensis* and *C. tropicalis*. The study encourages the application of locally selected yeast strains to benefit from converting starchy waste into useful products.

Keywords: α-amylase, Amyloglucosidase, Amylolytic yeast, Starch degradation

INTRODUCTION

Amylases are enzymes of significant industrial applications. They represent approximately 30% of the world enzyme production (Djkrif-Dakhmouche et al., 2016). The wide range of applications for amylases includes foods, industrial fermentation, detergent, textile, paper, as well as pharmaceutical industries (Kandra, 2003; Gupta et al., 2003; Souza, 2010). The amylases can be produced from plants, animals, and microorganisms. However, the amylases of microorganisms have a broad spectrum of industrial applications as they are more stable than plant and animal amylases (Tanyildizi et al., 2005). Additionally, some of the major advantages of using microorganisms for the production of amylases are the economical bulk production capacity and the ease of manipulation to enzymes of desired characteristics. Currently, commercial starch chemical hydrolysis is almost completely replaced by microbial amylases (Souza, 2010). Within microorganisms, the yeasts are preferred to bacteria and moulds for amylases production due to ease of culturing and safety (Fossi et al., 2005). And in recent years, researches were intensified on amylolytic yeast (Moubasher et al., 2010; Ouédraogo et al., 2012). Researchers have recently found some yeast strains have the ability to produce amylases, however, none specifically covers yeast amylases (Gupta et al., 2003; Djkrif-Dakhmouche et al., 2016). Previously, *Candida sake* HA124, *Candida tropicalis* HA147, *Candida membranifaciens* HA19 and *Cryptococcus heimaeyensis* HA7 were selected in our laboratory for their highest amylolytic activity during the screening of 198 isolates of yeast from different sources, namely, bagasse, wastes of potato chips manufacturing, bread dough, fermented local products (e.g. kishk and bosa), the surfaces of various types of fruits (e.g. apple, grape, plum and fig), and rotten fruits collected from local markets (Abdel-Raheam et al., 2011).

There is currently high interest in developing amylases for specific industrial applications such as raw starch degrading capacities with cost effective production techniques (Sivaramakrishnan et al., 2006). Amylolytic enzyme, such as α-amylase hydrolyzes soluble starch at α1,4 glucosidic bonds, producing maltose and maltotriose, while pullulanase hydrolyzes soluble starch at α1,6 glucosidic bonds revealing maltotriose only (Moubasher et al., 2010). Growth of the microorganism and enzyme production

are both strongly influenced by medium composition and culture parameters (Djekrif-Dakhmouche et al., 2006; Kathiresan and Manivannan, 2006). Therefore, the optimization of fermentation conditions (e.g., physical and chemical parameters) is at the heart of the cost-efficacy of the production process (Francis et al., 2003). The choice of appropriate carbon and nitrogen sources, as well as other nutrients, is a critical factor in the development of an efficient and economic process (Konsoula and Liakopoulou-Kyriakides, 2007). Temperature, incubation time, Inoculum size and pH are also significant environmental factors known to affect the microbial growth and enzyme production. Therefore, the present work was carried out to investigate certain nutritional and environmental factors affecting amylases production (α-amylase and AMG) by previously selected yeast isolates, in order to maximise the economic production of these enzymes.

MATERIAL AND METHODS

Procurement of strains

Four strains of amylolytic yeast namely, *Cryptococcus heimaeyensis* HA7, *Candida membranifaciens* HA19, *Candida tropicalis* HA147 and *Candida sake* HA124 isolated in our lab from starchy agro-industrial wastes and identified at Assiut University Microbiological Center (AUMC) were used for the optimization studies in this work.

Inoculation culture media

The medium used was the YM agar which consists of 10g D (+) glucose, 5g peptone, 3g yeast extract, 3g malt extract, and 15g agar agar per litre and the pH was adjusted to pH 3.5. This medium was used for maintenance, cultivation and activation of yeast strains. The inoculum was prepared by growing the culture in fresh YM broth medium and incubation at 30°C for 24h (Kumar and Satyanarayana, 2001).

Optimized culture medium (OPT)

For amylolytic enzymes production, the following medium was used: In (g/l), soluble starch, 5.0; yeast extract, 5.0; K2HPO4, 0.5; MgSO4.7H2O, 0.2; CaCl2.2H2O, 0.1; and pH was adjusted to 5.5 after autoclaving at 121˚C for 15 min (Gogoi et al., 1987). 50 ml of amylases production medium were dispensed into 250 ml Erlenmeyer flask, then autoclaved at 121°C for 15 minutes. After cooling at room temperature, each flask was inoculated with 1% of the culture suspension and then incubated. The optimized parameters for the environmental; incubation temperature, pH, inoculum size, incubation period and nutritional; carbon source and nitrogen sources factors were as shown in Table 1.

Assay of enzymes activities

At the end of the incubation period of the above treatments, the contents of each flask were centrifuged at 8000 rpm for 15 min at 4˚C and filtered through Whatman No.1 filter paper. The supernatant was collected as crude enzyme extract which used for determination of extracellular amylases activity.

Activity of α-amylase

The activity of α-amylase in culture fluids was measured by iodine method as described by Hernandez and Pirt (1975) as follow: 2.5ml of 0.4% soluble starch in phosphate buffer, pH 7.0 was mixed with 0.5 ml enzyme and incubated for 15 min at 50 °C. The reaction was stopped by adding 1.0 ml of 1.0 N HCl. 0.5 ml from each reaction tube was mixed with 1.0 ml of a (0.2% iodine-0.4% potassium iodide) solution and 2 ml distilled water, allowed to stand 15 min at room temperature and colour intensities were measured at 620 nm using Shimadzu (UV-visible-1601PC) spectrophotometer. The blank sample contained no starch and the control sample contained no enzyme. One unit of α-amylase activity was defined as the amount of enzyme, caused decrease of optical density by 0.05 in starch iodine coloration under the assay conditions.

Amyloglucosidase activity

AMG activity was determined by the method of Ramadas et al. (1996). The assay mixture consisted of 0.5 ml 1% soluble starch as substrate, 0.4 ml 0.02M sodium acetate buffer (pH 4.0) and 0.1 ml enzyme. After incubation for 10 min at 60°C the reaction was stopped by adding 1.0 ml/3.5-dinitrosalicylic acid reagent and heating for 5 min in boiling water. After cooling, the total volume was made up to 6 ml with distilled water and the A540 measured using glucose as standard. One unit of amylase activity was defined as the amount of enzyme causing release of reducing sugars equivalent to 1µM of glucose from starch per min under the assay conditions (Li *et al*., 2007b).

Statistical analysis

The statistical analysis of data was performed using GraphPad Prism, release 6. ANOVA followed by Tukey-HSD tests for multiple compression was used to compare different treatments at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Environmental factors

Effect of incubation temperature (TrE1)

Incubation temperature influences not only the growth of microorganisms, but also their biological activities. Therefore, an experiment was conducted to find out the effect of different incubation temperatures (20, 25, 30 and 35˚C) on production of α-amylase and amyloglucosidase by *Cryptococcus heimaeyensis*, *Candida membranifaciens*, *Candida tropicalis* and *Candida sake*. As shown in Figs $1_{A,B}$, the production of α-amylase and amyloglucosidase peaked at 25 ˚C for all yeast strains. The results showed that there were significant differences in the effect of incubation temperature degrees of all tested yeast strains (P = < 0.0001), as well as within the different temperatures of incubation (P = < 0.0001). A closer look at the results showed that under various incubation temperatures of α-amylase production, no significant difference among all tested yeast strains was observed (P = 0.5671). On the other hand, the results showed significant differences in the activity of the α-amylase enzyme with different incubation temperatures for within each yeast strain (P = <0.0001). The AMG enzyme activity was highest at 25 °C and significantly different from other temperatures. The mean values were 21.8 ±2.1, 22.7±1.7, 24.0±1.8 and 30.5±2.0 for *Cry. heimaeyensis*, *C. membranifaciens*, *C. tropicalis* and *C. sake*, respectively. The *C. sake* strain had the highest activity of AMG at 25 °C. These results are somewhat at variance with those of Sandhu et al. (1987) and Li et al. (2007a) who found that the optimal temperature for α-amylase production by *Saccharomycopsis fibuligera* and *Aureobasidium pullulans* yeast strains was 28 ˚C. On the other hand, best amylolytic enzymes production by *Saccharomycopsis fibuligera* ranged from 30°C to 40°C in batch culture mode as reported by Gonzalez et al. (2008).

Effect of initial pH values (TrE2)

The pH is one of the important factors for all microbial industries. This importance is due to its impact on both the growth and activity of microorganisms. The effect of varying pH from 4.0 to 6.5 on α-amylase and AMG activities of tested yeast strains was investigated. The results are depicted in Figs $2_{A,B}$ indicated that the production of α- amylase and AMG were found to be the optimum at pH 4.5 for both *C. membranifaciens* and *C. tropicalis*. On the other hand, pH 6.0 was the optimum for all enzymes production by *Cry. heimaeyensis* and *C. sake*. Furthermore, no significant differences between *C. membranifaciens* and *C. tropicalis* for all enzyme activities (p=>0.05). Therefore, a pH regulatory system is particularly important since the production of extracellular enzymes is known to be pH dependent (Denison, 2000). The acidic medium was required for optimum production of all amylase enzymes which produced by tested yeast strains. Similar findings have been previously reported by Sandhu *et al*. (1987) and Hostinová (2002) who found that optimal pH for α-amylase production by *Saccharomycopsis fibuligera* ranged from 5.0 to 6.2. Using a recombinant *Saccharomyces cerevisiae* strain YPG/AB, Ülgen et al. (2002) reported that the optimum pH for glucoamylase and α-amylase production were 4.0–4.5 and 5.0–7.0, respectively in YPS medium. Also, Gupta et al. (2010) recorded that enzyme production started at pH 3.0 and ceased at pH 8.0. Additionally, the maximum enzyme production occurred at pH 4 to 6 and very little growth was observed without enzyme production in medium at initial pH 3 to 4.

Effect of inoculum size (TrE3)

As the inoculum size is known to affect the growth rate and hence microbial activity, its effect was also studied at levels 0.5, 1, 2 and 3%, v/v. As shown in Figs $3_{A, B}$, the rate of α-amylase and AMG activities were optimum at 1% inoculum size for all tested yeast strains. Therefore, 1% was selected as best economical inoculum size for all tested yeast strains in further experiments. These findings were consistent among all strains and enzymes despite significant differences on their activity levels at the same inoculum size. At low inoculum size, the cell numbers are probably too low to produce an optimum active numbers of yeast cells while the high inoculum size led to inter-competitive cell activity and consumption the major portion of the substrate affecting metabolic process of enzyme synthesis, hence lower enzyme activity (Abdullah et al., 2014).

Effect of incubation period (TrE4)

Incubation period plays an important role in substrate utilization and enzyme production. The effect of incubation period was evaluated by checking enzyme activities after 12, 24, 48, 72, and 96 h of incubation (Figs $4_{A, B}$). The results of these experiments indication the maximum enzyme activity is reached after 48 h for *C. sake*, *C. tropicalis*, and *C. membranifaciens*, but after 72 h for *Cry. heimaeyensis*. The decreasing of amylolitic enzyme activities after reaching a maximum incubation time is perhaps due to the denaturation of the enzyme caused by the interaction with other components (by-

Table 1. Different treatments of environmental and nutritional parameters were used to maximize the amylase enzymes

Treatments	Environmental factors				Nutritional factors	
	Incubation temperature °C	pH	Inoculum size %	Incubation period (h)	Carbon source	Nitrogen source
TrE1	20-35	5.5	1.0	48	Starch[1]	Yeast extract[2]
TrE2	30	4- 6	1.0	48	Starch[1]	Yeast extract[2]
TrE3	30	5.5	0.5-3	48	Starch[1]	Yeast extract[2]
TrE4	30	5.5	1.0	12- 96	Starch[1]	Yeast extract[2]
TrN1	30	5.5	1.0	48	Starch, Maltose, Sucrose, Glucose (0.5%)	Yeast extract[2]
TrN2	30	5.5	1.0	48	Starch[1]	Peptone, Yeast extracts, Beef extract, Ammonium sulphate (0.5%)
TrN3	30	5.5	1.0	48	Starch: 0.5-6.0 %	Yeast extract[2]
TrN4	30	5.5	1.0	48	Starch[1]	Yeast extract @ 0.5 -3 %

Where, the environmental treatments: TrE1, TrE2, TrE3 and TrE4 of incubation temperature, pH, inoculum size and incubation period, respectively. The nutritional treatments: TrN1 = carbon sources, TrN2 = nitrogen sources, TrN3 = starch concentrations, TrN4 = yeast extract concentrations.[1]starch concentration = 5.0 g/l, [2]yeast extract concentration = 5.0 g/l.

Table 2. Effect of carbon and nitrogen sources on α-amylase and Amyloglucosidase production by tested yeast strains (U/mL)

	Cry. heimaeyensis		*C. membranifaciens*		*C. tropicalis*		*C. sake*	
	α-A	AMG	α-A	AMG	α-A	AMG	α-A	AMG
Carbon source								
Glucose	13.6±1.4[a]	10.3±1.6[a]	13.7±1.6[a]	10.1±1.1[a]	13.5±1.4[a]	11.2±1.7[a]	10.6±1.1[a]	10.6±0.8[a]
Sucrose	14.2±2.1[a]	11.1±1.0[a]	14.3±2.1[a]	11.2±1.7[a]	13.8±2.1[a]	11.5±1.1[a]	10.8±1.7[a]	11.3±1.1[a]
Maltose	14.3±1.7[a]	11.4±2.3[a]	15.5±2.0[a]	12.9±2.0[a]	21.4±2.3[b]	23.3±2.4[b]	18.4±2.7[a]	22.7±1.3[b]
Starch	26.2±2.1[b]	29.9±3.3[b]	25.3±2.7[b]	28.1±3.0[b]	25.8±3.4[b]	30.1±2.5[b]	26.0±1.6[b]	34.4±2.8[c]
Nitrogen sources								
Yeast extract	40.1±5.8[a]	51.1±4.4[a]	43.1±6.3[a]	55.6±6.5[b]	47.9±8.3[a]	101.6±9.3[b]	43.8±5.4[a]	97.8±8.2[b]
Beef extract	37.4±7.7[a]	47.0±7.1[a]	32.9±4.1[a]	39.9±4.1[a]	40.7±5.2[a]	53.1±4.4[a]	30.4±3.4[a]	41.0±4.2[a]
Ammonium sulfate	34.1±5.9[a]	41.9±5.5[a]	34.9±9.8[a]	42.9±2.7[a]	37.2±7.4[a]	47.7±8.1[a]	32.3±4.7[a]	44.1±5.8[a]
Peptone	31.3±6.9[a]	37.5±3.5[a]	35.0±8.2[a]	43.1±3.7[a]	36.6±3.7[a]	46.7±3.8[a]	28.5±6.4[a]	38.2±6.8[a]

Values are mean ± SD of duplicate observations. Values which are significantly different at α=0.05 are marked with different letters; multiple comparisons tests were performed within each nutrient source.

Table 3.Optimized environmental and nutritional parameters for the production of α-amylase and amyloglucosidase (AMG) enzymes by selected yeast strains

	Cry. heimaeyensis		*C. membranifaciens*		*C. tropicalis*		*C. sake*	
	α-A	AMG	α-A	AMG	α-A	AMG	α-A	AMG
Environmental factors								
Incubation Temp. (°C)	25	25	25	25	25	25	25	25
pH	6	6	4.5	4.5	4.5	4.5	6	6
Incubation Size (%)	1	1	1	1	1	1	1	1
Incubation Period (h)	72	72	48	48	48	48	48	48
Nutritional factors								
Carbon source	Starch	Starch	Starch	Starch	Starch	Starch	Starch	Starch
Nitrogen source	Yeast extract	Yeast extract	Yeast extract	Yeast extract	Yeast extract	Yeast extract	Yeast extract	Yeast extract
Starch (%)	4	4	4	4	4	4	5	5
Yeast extract (%)	1	1	1	1	2	2	1	1

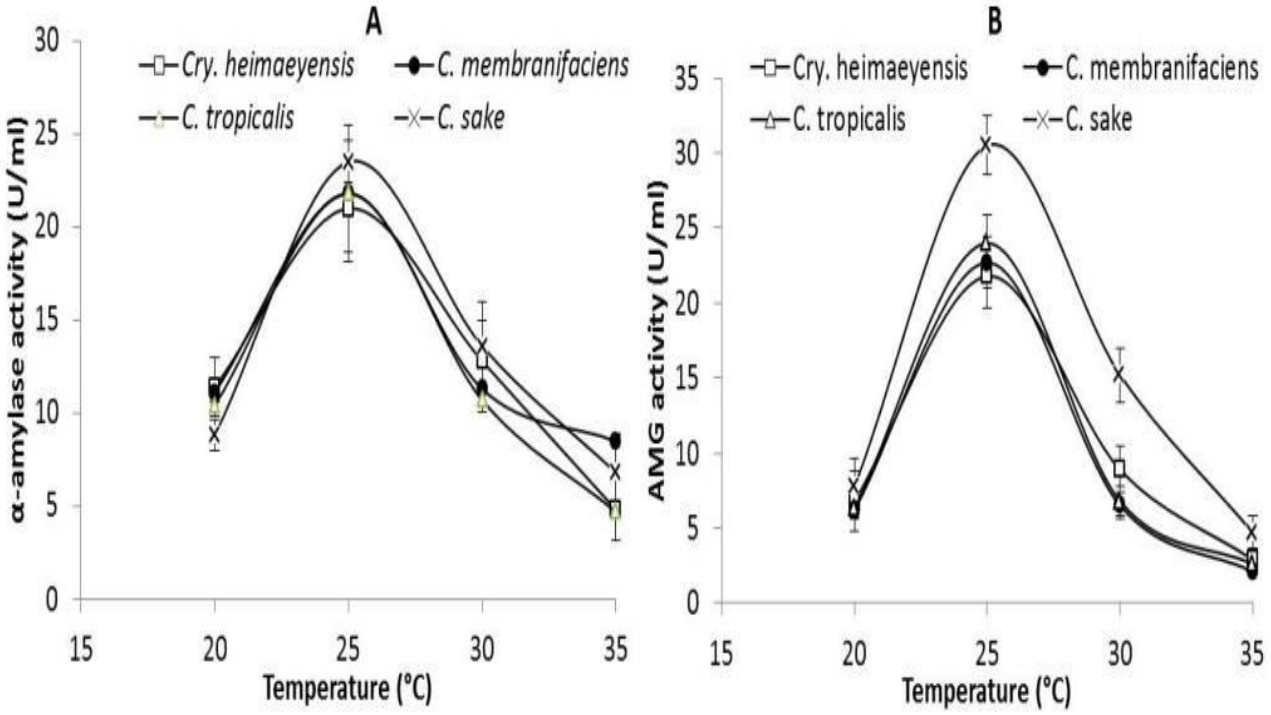

Fig 1. Effect of incubation temperature on α-amylase (A) and AMG (B) production by tested yeast strain

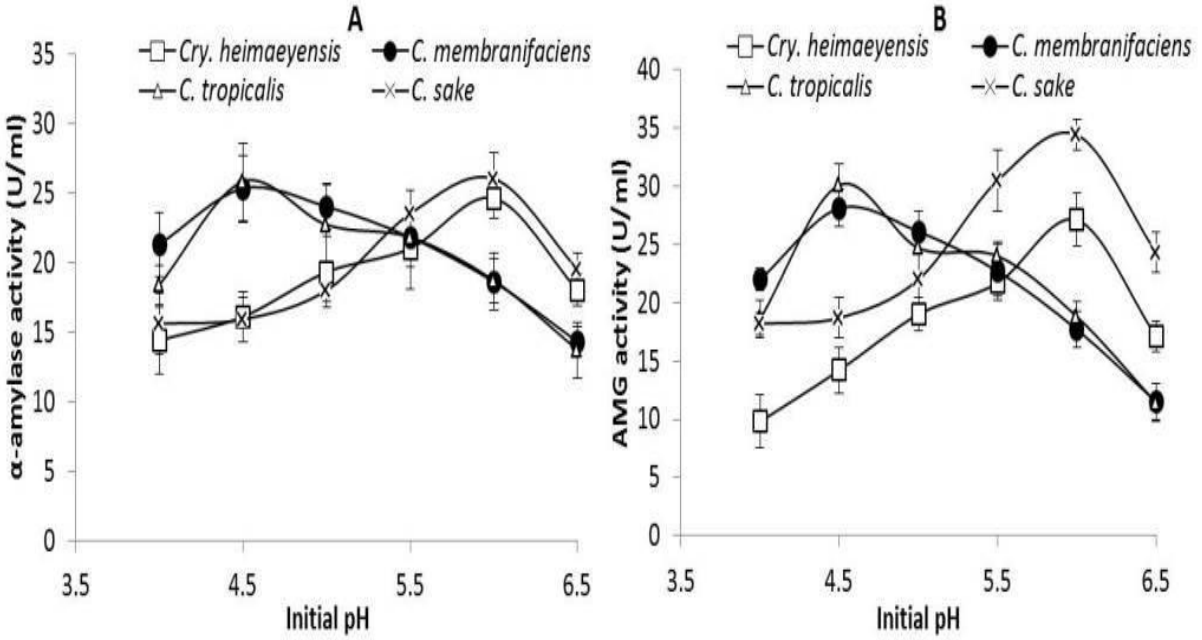

Fig 2. Effect of initial pH values on α-amylase (A) and AMG (B) production by tested yeast strains

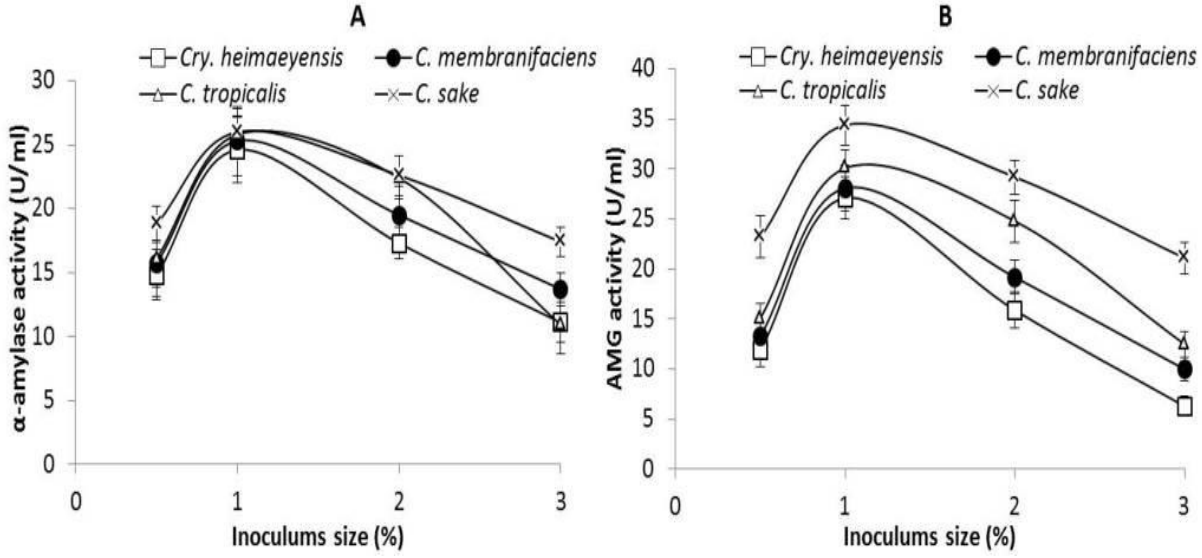

Fig 3. Effect of inoculum size on α-amylase (A) and AMG (B) production by tested yeast strains

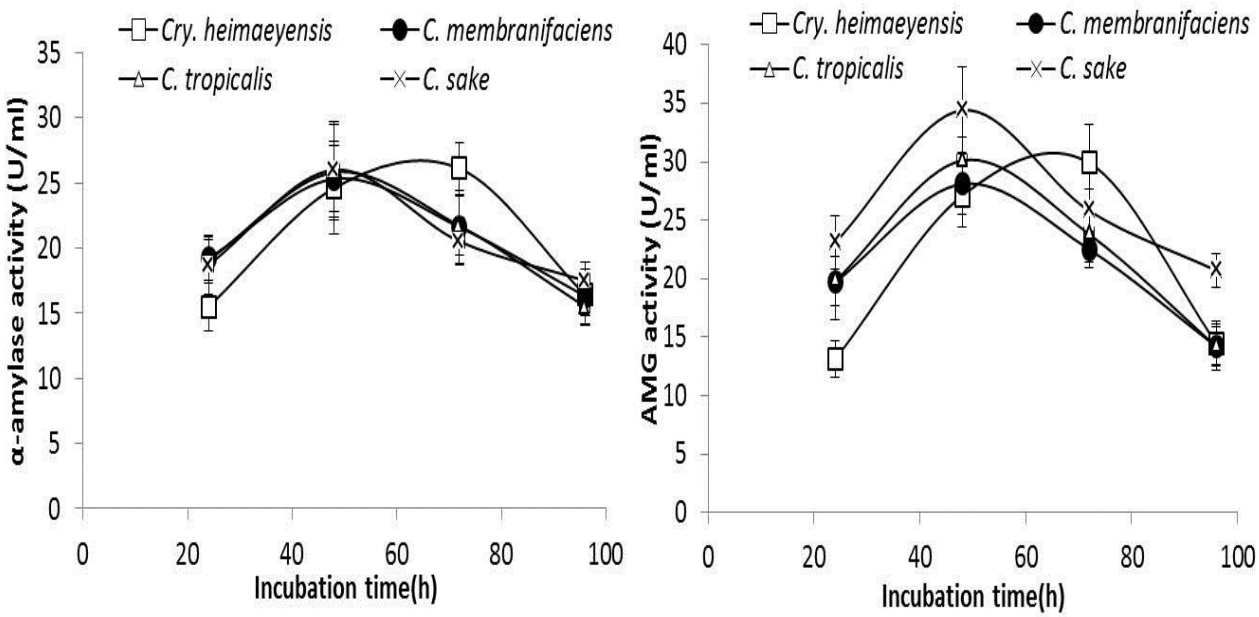

Figure 4. Effect of incubation period on α-amylase (A) and AMG (B) production by tested yeast strains

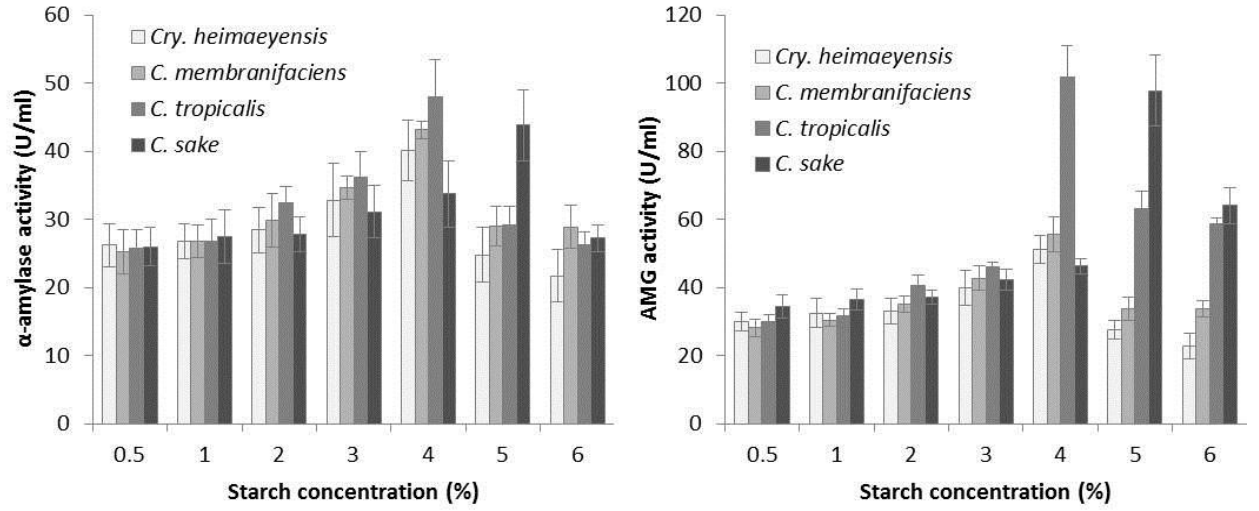

Fig 5. Effect of different starch concentrations on α-amylase (A) and AMG (B) production by tested yeast strains

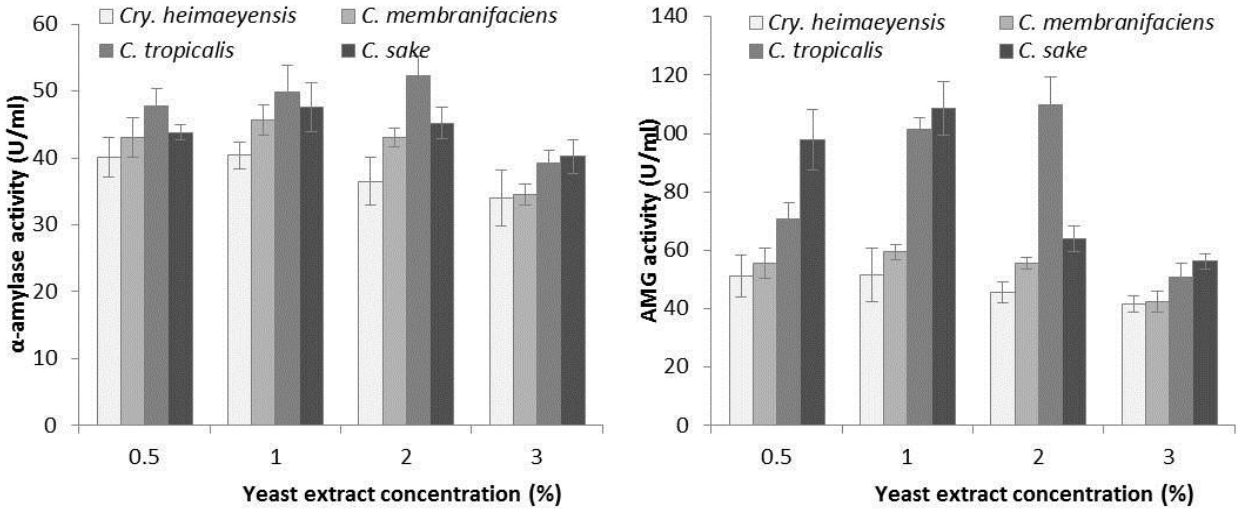

Fig 6. Effect of different yeast extract concentrations on α-amylase (A) and AMG (B) production by tested yeast strain

products) in the medium and/or the depletion of essential nutrients available to microorganism. This interpretation corresponds to similar studies for the production of certain amylase enzymes by the various strains of Fungi (Abdullah et al., 2014; Gupta et al., 2010; Ramesh and Lonsane, 1987; Krishna and Chandrasekaran, 1996). Previous findings reported that almost all starch was hydrolysed after 48 h of incubation on *Sympodiomyces attinorum*, *Saccharomyces diastaticus* and *Endomycopsis capsularis* strains (Alonso et al., 2010; Fossi et al., 2005; Verma et al., 2000). Our study is the first to show that the enzyme activity can be still be increasing up to 72 h for some yeast strains (Cry. heimaeyensis).

Nutritional factors

Carbon sources (TrN1)

This experiment was designed to investigate the effect of various carbohydrates as sole carbon source on α-amylase and amyloglucosidase (AMG) production by the four tested strains. Starch, maltose, sucrose and glucose were tested separately at concentration of 0.5% of each at the optimum environmental factors (Table 1). The results presented in Table 2 illustrated that starch, followed by maltose, were the best carbon sources for α-amylase and AMG productivities. Data also showed that there were significant differences in the activity of all tested enzymes between the starch and the other carbon sources tested. Glucose and sucrose experiments recorded that there were no significant differences in the enzyme activities among all the studied yeast strains. The same results were observed by starch experiment. While in the cases of maltose treatment, there were significant differences among some yeast strains for AMG activity. This result confirms previous findings by (Kumar and Satyanarayana, 2001; De Mot, 1987) where maltose, dextrins and starch were strong inducers of yeast amylases. It is because amylases are extracellular enzymes and their productions are increased by their substrates, furthermore, for their inductive effect and their roles in stabilizing the enzymes (De Mot and Verachtert, 1987; Djkrif-Dakhmouche et al., 2014). In contrast, many researchers recorded a decline in the production of α-amylase when adding starch. This inhibition of yeast growth and amylolytic activity may occur due to limitation of other media components in the culture medium. Moreover, Boze et al. (1989) reported a repression in α-amylase activity in the yeast *Schwanniomyces castellii* strain CBS 2863 in the presence of glucose.

Nitrogen sources (TrN2)

The effect of different nitrogen sources such as yeast extract, beef extract, ammonium sulphate and peptone on the production of α-amylase and amyloglucosidase were investigated. As shown in Table 2, there were significant differences in the activity of AMG enzyme between yeast extract and other nitrogen sources. That was observed in all yeast strains except genus *Cry. heimaeyensis* where no significant difference was observed. Yeast extract proved to be the best nitrogen source as it revealed

the highest production of α-amylase and amyloglucosidase in most tested yeast strains. The results indicated that there were significant differences of AMG among *Cry. heimaeyensis* and *C. membranifaciens* on one hand and the *C. tropicalis* and *C. sake* on the other hand when yeast extract was the nitrogen source (alpha = 0.05). It was observed that inorganic nitrogen (as ammonium) and peptone negatively affected amylase enzyme biosynthesis by *Saccharomycopsis fibuligera*, whilst yeast extract proved to be highly effective as an organic nitrogen source with no interference on the amylolytic enzymes production profile (Gonzalez et al., 2008). On the other hand Djkrif-Dakhmouche et al. (2014) reported the yeast extract had a significantly negative effect on the α-amylase production. They explained this result was may be due to the excessive amount of the yeast extract, which causes inhibition of the enzyme production when concentration exceeds the critical value.

Effects of starch concentrations (TrN3)

From the previous experiment (TrN1), starch secured maximum yield of α- amylase and amyloglucosidase (AMG) when used as a sole carbon source for *Cry. heimaeyensis*, *C. membranifaciens*, *C. tropicalis*, and *C. sake*. Therefore, this experiment was designed to identify the optimum starch concentration that could stimulate the enzymes production by the same yeast strains. Starch was added to the amylase production medium at concentrations of 0.5, 1, 2, 3, 4, 5 and 6% (w/v). As shown in Figs $5_{A,B,}$ the accumulation of α-amylase and Amyloglucosidase (AMG) increased gradually as the starch concentration increased reaching the maximum at 4% concentration for *Cry. heimaeyensis*, *C. membranifaciens* and *C. tropicalis* but at 5% for *C. sake*. The results showed there were significant differences in α-amylase productions at 5% starch concentration between *C. sake* and other tested yeast strains. Additionally, at 4, 5 and 6% starch concentrations , significant differences were observed in the AMG production among most yeast strains (alpha= 0.05). These results are somewhat at variance with those of Alonso et al. (2010) who selected (5%) starch concentration as the most economical, and the best concentration for the production of high amylase (s) by *Sympodiomyces attinorum*. However, the results are consistent with those reported by Ülgen et al. (2002) who indicated that glucoamylase activity increased by a recombinant *saccharomyces cerevisiae* strain YPG/AB with an increase in initial starch concentration in the medium. On the other hand, Sandhu et al. (1987) found that the optimal soluble starch concentration for α-amylase production by *Saccharomycopsis fibuligera* yeast strain was 1.5% w/v. These varied findings may be attributed to the variance in the fermentation conditions or to the use of different yeast cultures. The increase in carbon source concentration over a certain extent might have caused catabolic repression (Gupta et al., 2008).

Effects of yeast extract concentrations (TrN4)

During the experiment TrN2, it was observed that yeast extract was the best source of nitrogen in terms of the production of amylolytic enzymes. Therefore, the effects of different yeast extract concentrations, (0.5, 1, 2 and 3%) on the amylolytic enzymes production was investigated. As shown in Figs $6_{A,B}$, the maximum amylolytic activities took place at the concentration of 1% for *C. sake*, *C. membranifaciens* and *Cry. heimaeyensis* while it was at 2% for *C. tropicalis*. At the optimum concentration of yeast extract (1%), no significant differences in the production of α-amylase among yeast strains were observed, while in the production of AMG enzyme, the strains *C. tropicalis* and *C. sake* showed superiority compared to the other yeast strains. Similar results were obtained by Punpeng et al. (1992) who used 1% yeast extract in the production medium for the production of glucoamylase by *Saccharomycopsis fibuligera*, *Schwanniomyces alluvius* and *Lipomyces starkeyi*. The yeast extract is an important source of nitrogen and intake of essential vitamins group B), particularly favorable to the growth of most microorganisms (Spencer-Martins and Van Uden, 1979).

Technological impact

The conducted experiments in this work can be exploited in the technological applications related the amylolytic enzymes production by selected yeast strains (*Candida sake* HA124, *Candida tropicalis* HA147, Candida membranifaciens HA19 and *Cryptococcus heimaeyensis* HA7). These strains were selected based on the screening of 198 isolates of yeast from different sources, namely, bagasse, wastes of potato chips manufacturing, bread dough, fermented Egyptian products (e.g. kishk and bosa), the surfaces of various types of fruits (e.g. apple, grape, plum and fig), and rotten fruits collected from local markets. The environmental and nutritional factors were optimized to yield the highest production of α-amylase and amyloglucosidase (AMG). These thermostable amylolytic enzymes differ in their optimum pH, temperature, temperature stability and in other several physiochemical properties depending on the species origin, and hence different enzymes have found specific applicability in

different industries (Gupta et al., 2010). Thus, the optimization of these environmental and nutritional parameters is very essential for the production of amylase enzymes. A summary of this optimization is shown in Table 3.

CONCLUSION

The starchy waste either agricultural or industrial poses a difficult environmental problem. Therefore, this study focused on optimizing the environmental and nutritional conditions to obtain higher productivity of amylase enzymes by the yeast strains isolated from the indigenous sources. The results showed that the strain *C. sake* was the best-yielding in the production of amylase enzymes, especially AMG enzyme compared with other yeast strains under the same environmental conditions. The optimal incubation temperature, pH, inoculum size and incubation period were 25°C, 6, 1% and 48h, respectively. On the other hand, the starch and yeast extract gave higher production for all studied enzymes compared with other carbon and nitrogen sources. The optimum starch concentration for *C. sake* was 5% while it was 4% for other yeast strains. In addition, the optimum yeast extract concentration (nitrogen source) was 1% for most studied yeast strains but at 2% for *C. tropicalis*.

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Comparative study of date and fenugreek seeds phenolics properties

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ABSTRACT

Date and fenugreek seeds contain phenolic compounds, which have antioxidant and antibacterial properties. In this study, antioxidant and antibacterial potentials of date and fenugreek seeds were determined. The antioxidants were extracted by using aqueous treatment, 50% methanol, 50% ethanol, 50% acetone and 50% dichloromethane. The results demonstrated that ethanol extracts of date and fenugreek seeds showed the highest TPC contents which were accounted as 2404mg GAE/100g and 959.05mg GAE/100g, respectively. The highest DPPH activities of date and fenugreek seeds were 76.15% and 62.97%, respectively. Similarly, ethanol extracts of both seeds showed the highest antimicrobial activity against *E.coli*. The phenolic acids like gallic, coumeric, syringic, sinapic, vanillic, chlorogenic, ferulic and benzoic acids were determined with high performance liquid chromatography (HPLC).

Key words: antioxidant, phenolic acids, antimicrobial, activity, date seeds, fenugreek seeds

INTRODUCTION

The date *(Phoenix dactylifera L.)* an energy rich fruit, is regarded as a popular food for thousands of years in the Arabian Gulf peninsula and also in its neighboring region (Khatchadourian *et al.*, 1982). It is a major fruit crop of the Middle East countries. About 75 to 80% of the world production is produced in this region (Yousif *et al.*, 1990). Dates are sweet and palatable eaten as a staple food by the population of dates producing countries (Sidhu, 2012). Weight of date seed ranges from 0.5 - 4g, the width is 0.6 - 1.3cm and the length 1.2 -3.6cm. It contains hard endosperm that is made up of cellulose present on the inner side of the cell wall (Zaid, 2002). Almana and Mahmoud (1994) reported that date seeds represent about 10% of the fruit. They are usable as a substitute source of dietary fiber as compared to bran of wheat, because they contribute to the consumption of dietary fiber. Therefore, the seeds were examined because they showed the great value for extractable components for including in functional foods. Some minor components were extracted from the seeds such as alkali soluble heteroxylan and water soluble galactomannan (Ishrud *et al.*, 2003). It is reported that they can also be used as a source of oil, as a coffee substitute and as a raw material for activated carbon or as an adsorbent for water containing dye (Banat *et al.* 2003; Barreveld, 1993). They are traditionally used for animal feed. Vandepopuliere *et al.* (1995) recommended the addition of the seeds in diet of broilers at different levels ranging from 5 to 27%. Date fruits and their seeds have been used in the different conventional and folk systems of medicines (Khare, 2007). An ethno-medicinal survey conducted in the Morocco has shown that dates are conventionally used to treat diabetes and hypertension (Tahraoui *et al.*, 2007). These are suitable for the food preparation and fiber-based dietary supplements because of their higher dietary fiber content. The dietary fiber showed therapeutic effect for certain conditions such as higher cholesterol, diabetes, obesity and also showed the preventing effect against coronary heart disease, hypertension, prostate, intestinal disorders and colorectal cancers (Kritchevsky, 1988; Johnson and Southgate, 1994; Tariq *et al.*, 2000).

Fenugreek belongs to legume family and grown mostly as a spice in the world. It has a prolonged history as both culinary and medicinal herb in the ancient world. The spice name *"foenumgraecum"* means "Greek Hey" indicates that it is used as a forage crop world widely (Acharya *et al.*, 2006). It is mostly cultivated in Egypt, India, Pakistan and Middle Eastern countries. Both the seeds and leaves are used as spice and green vegetable, respectively (Youssef *et al.*, 2009). Raw seeds contain maple

flavor and bitter taste but bitterness can be reduced by the roasting process and therefore the flavor is improved. These seeds are mainly used as spices such as powder is used in marinades and vegetable dishes. These seeds are fibrous, gummy and sticky in nature and are used as condiments. Seed is the most utilized part of fenugreek and much popular in snacks and savory recipes and also has medicinal importance (Zargari, 1988; Khosla *et al*., 1995). Due to its fiber, protein and gum content, it is used as food emulsifier, adhesive and as stabilize. Alkaloids and saponins are anti-nutrients found in fenugreek (Meghwal and Goswani, 2012). The effect of fenugreek seeds was studied on ethanol-induced gastric ulcer. Ulcer protective effects have been shown by gel fraction and aqueous extracts of fenugreek seeds. Anti-secretary action was not only involved to produce cytoprotective effect but it also has effect on mucosal glycoprotein. Muscosal injury is also lowered by preventing the lipid peroxidation and by enhancing antioxidant potential. It was notified from histological studies that soluble gel obtained from fenugreek seeds has more effectiveness to prevent lesion formation. These all findings indicated that fenugreek seeds have a good antiulcer effect (Pandian *et al*., 2002). In this study, antioxidant and antibacterial potentials of date and fenugreek seeds were determined.

MATERIALS AND METHODS

Procurement of raw materials

Date and fenugreek seeds were procured from local market. Analytical grade chemicals were purchased from Sigma-Aldrich, Germany. The research was conducted in the laboratories of National Institute of Food Science and Technology, University of Agriculture, Faisalabad–Pakistan.

Extraction of phenolic compounds

1.5g of sample was taken in a conical flask and then 60mL solvent was added in each sample (aqueous extract, 50% methanol, 50% ethanol, 50% acetone and 50% dichloromethane) and then stirred at the rate of 150 rpm with magnetic stirrer on a hotplate at 45°C for 2hrs. Then it was filtered through Whatman filter paper No. 4 and concentrated by using rotary evaporator. (Al-Farsi and Lee. 2008).

Yield estimation

3mL of each extract were measured into a pre-weighed aluminum dish. The samples were kept in an oven at 85°C for 24h followed by placing in a desiccator for 12h. The weight difference was used to calculate percentage yield (Prashani *et al*., 2005).

Phenolic content

Total phenolic contents (TPC) in date and fenugreek seed extracts were quantified by using Folin-Ciocalteu method (Iqbal *et al*., 2005) that was based on the reduction of phosphotungstic acid to phosphotungstic blue and as a result, absorbance increased due to rise in number of aromatic phenolic groups. For the purpose, 200µL of sample were separately added to test tube containing 1mL of Folin-Ciocalteu's reagent, 2mL of 7.5% sodium carbonate solution and volume was made up to 7mL with distilled water. The absorbance of the resulting blue color was measured at 765nm on UV/visible light Spectrophotometer. Total polyphenols was estimated and values were verbalized as gallic acid equivalent (mg gallic acid/g).

Total phenolic compounds of each sample in gallic acid equivalents (GAE) was calculated by following formula:

$$C = c \times V / m$$

C = Total phenolic contents (mg/g plant extract, in GAE)

c = Concentration of gallic acid (mg/mL)

V = Volume of extract (mL)

m = Weight of sample (g)

Free radical scavenging activity (DPPH assay)

DPPH (1,1-diphenyl-1-picrylhydrazyl) is a stable and highly colored oxidizing radical that result in formation of a yellow colored hydrazine (DPPH-H) associated with abstraction of free hydrogen atoms from phenolic antioxidants. Protocol of Tadhani and Subhash, (2007) was followed to determine DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical scavenging activity of date seed and fenugreek seed extracts. 4mg of DPPH was dissolved in 100 mL methanol and 2 mL of this solution was added to 50µL sample extract. The mixture was shaken vigorously and allowed to stand at room temperature in a dark place. Then the absorbance was measured at 515 nm. The radical scavenging percentage was calculated using the following equation. The free radical-scavenging activity of each sample can be presented as percentage reduction in DPPH due to given amount of each extract.

Reduction of absorbance (%) = [(AB – AA) / AB] × 100

AB = Absorbance of blank sample at t = 0 min

AA = Absorbance of tested extract solution at t = 15min

Flavonoids

Protocol of Tadhani and Subhash (2007) was followed to determine the flavonoid contents in date seed and fenugreek seed extracts. Briefly, 1mL sample was added to a 10mL of volumetric flask containing 4mL of distilled water followed by immediate addition of 0.6mL of 5% $NaNO_2$, 0.5mL of 10% $AlCl_3$ after 5min, and 2mL of 1M NaOH after 1min. Furthermore, each reaction flask was then immediately diluted with 2.4mL of distilled water and mixed. The absorbance of pink colored solution was noted at 510nm. The quercetin (µg/g) was used as a standard for the calibration curve. The total flavonoid content of the samples was calculated by using the calibration curve.

Determination of individual phenolic acids

Sample Preparation for HPLC

Fifteen grams of each sample were taken in the flasks and 600mL of solvent were added in each flask and heated on a hot plate. Samples were highly concentrated after heating. A rotary evaporator was used for evaporation of the solvent. Then 5mL extract of each sample were taken in a flask, followed by adding 6mL HPLC grade water and 12mL methanol. It was shaken for 5 min and then 1mL of 6M HCl was added. Then the flasks were placed on a water bath for 2h at 50-60ºC. The sample was filtered by using 0.22µm syringe filter and placed in eppendorf. The concentration of phenolic in unknown samples was calculated as:

$$\text{Concentration of unknown} = \frac{\text{Peak area of sample} \times \text{Conc. of standard}}{\text{Peak area of standard}}$$

Determination of individual phenolic acids

Individual phenolic acid in the extracts was analyzed by a Hewlett-Packard 1100 Series high-performance liquid chromatograph equipped with UV detector (Hewlett-Packard, Palo Alto, CA) and Phenomenex Jupiter C18 (250-4.60mm2, 10mm, 300 A; Phenomenex, Torrance, CA) column. The mobile phase of water with 0.05% trifluoroacetic acid (solvent A) and 30% acetonitrile, 10% methanol, 59.95% water and 0.05% trifluoroacetic acid (solvent B) was used at a flow rate of 1.0mL/min. Total run time was 50min and the gradient program was as follows: 10–12% B for 16min, 12–38% for 9 min, 38–70% B for 7min, 70–85% B for 8min and 85–100% B for 10min. There was 5min of post-run for reconditioning. The injection volume was 20mL. Detection was done at 280nm via UV detector. Identification and quantification of phenolic acids in samples were performed by comparing the chromatographic retention times and areas of external standards. Phenolic acid standards used for peak identification, ferulic acid, vanillic acid, syringic acid, gallic acid, p-hydroxybenzoic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid and trans-cinnamic acid and used without further purification (97% and higher purity). (Irmak *et al*., 2007).

Antimicrobial activity of extracts

The antimicrobial activity of date seed and fenugreek seed extracts against food borne pathogens *Escherichia coli* was tested by using following method.

Disc diffusion method

The antimicrobial activity of date seed and fenugreek seed extracts was determined by disc diffusion method as described by Mukhtar and Ghori, (2012). For this purpose, round disks (6mm) in diameter were cut out from disk paper. These disks were sterilized in autoclave and soaked with 20micro liters of each extract. The inoculum suspension of *Escherichia coli* was swabbed on the entire surface of Muller-Hinton agar (MHA). The sterilized paper discs, previously soaked with extract were aseptically placed on MHA surfaces. A standard antibiotic (Amoxillin) was used as standard. The plates were then incubated at 37ºC for 24h to check the growth of pathogens. The extracts having antimicrobial activity to inhibiting the bacterial growth were identified and clear zone formed was measured as zone of inhibition in millimeter. Each experiment was done in triplicate.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that prevents growth of microorganisms after a specific incubation period i.e. the lowest concentration at which no growth occurs in a nutrient medium following incubation. For the conduct of experiment, two fold serial dilutions were prepared

for each extract that was added into the kit. A control was also prepared with an antibiotic (Amoxicillin) which was used as a standard for comparison. The incubation was done at 37ºC for 24h and minimum inhibitory concentration (MIC) was determined. To each well, 10micro liters of resazurin indicator solution were added. The absorbance was measured by micro plate reader. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color changes occur was taken as MIC value (Mehmood *et al*., 2012).

RESULTS AND DISCUSSION

Yield of extracts with different solvents

The yield of the date and fenugreek seed extracts obtained by using rotary evaporator were calculated in percentages. Extracts of both seeds were prepared by using different solvents like aqueous, 50% methanol, 50% ethanol, 50% acetone and 50% dichloromethane. According to the polar nature and chemical composition of phenolic compounds extracts obtained from ethanol and methanol contained relatively high percentage yield as compared to the other solvents.

Yield of date seed obtained with 50% ethanol, 50% methanol, 50% acetone, 50% dichloromethane and aqueous were 36.83±0.63%, 36.19%±0.09, 18.72±0.22%, 14.24±0.35% and 10.53±1.63%, respectively (Table 1). Yields of fenugreek seed obtained with 50% ethanol, 50% methanol, 50% acetone, 50% dichloromethane and aqueous were 27.68±0.96%, 27.03±0.13%, 16.36±1.26, 11.76±0.57% and 8.93±0.31%, respectively (Table 1). These results are agreed with the findings of Bukhari *et al*. (2008) who reported that yields obtained with methanol and ethanol were found higher as compared to the other solvents like dichloromethane, acetone, hexane and ethyl acetate.

Total phenolic contents

The total phenolic compounds (TPC) of date and fenugreek seeds were expressed in terms of mg GAE/100g in Table 2. Date seed extracts obtained by using different solvents like 50% ethanol, 50% methanol, 50% acetone, 50% dichloromethane and aqueous contain 2404.8±167.64mgGAE/100g, 1671.0±58.06mgGAE/100g, 900.43±54.63mgGAE/100g, 765.33±38.67mgGAE/100g and 718.10±15.67mgGAE/100g, respectively. Fenugreek seed extracts contained 959.05±32.68mgGAE/100g, 739.12±22.01mgGAE/100g, 583.96±37.26mgGAE/100g, 295.89±20.19mgGAE/100g, 125.05±17.56mgGAE/100g obtained with 50% ethanol, 50% methanol, 50% acetone, 50% dichloromethane and aqueous samples, respectively.

Ardekani *et al*. (2010) determined the total phenolic compounds of 14 date seed varieties by using five different solvents including water, methanol, 50% methanol, DMSO and water: methanol: acetone: formic acid (20:40:40:0.1). They stated that all the extracts had considerable TPC ranging from 3541 mgGAE/100g in zahedi to 1260 mgGAE/100 g in seeds of shahabi variety. Previously, Al-Farsi and Lee (2008) reported that date seeds contained significant amount of phenolics ranging from 3102 to 4430 mg GAE/100g.

Bukhari *et al*. (2008) did an experiment to find total phenolic content of fenugreek extract by using various solvents. The findings of the analysis are found in close agreement to the present results. They showed that methanol and ethanol possessed the highest total phenolic contents such as 575±0.002 and 685±0.002 (mg GAE/100g), respectively. Other solvents contained less phenolic fractions as dichloromethane 227±0.003, hexane 135±0.002, and ethyl acetate 332±0.004 (mg GAE/100g).

Free radical scavenging activity (DPPH assay)

DPPH of date seed extracts is shown in Table 3. Radical scavenging activities of ethanol extract was found higher (76.15±2.19%) as compared to methanol (74.20±1.82%), Acetone (63.12±2.80%), dichloromethane (48.94±2.45%) and aqueous (42.10±1.73%). DPPH of fenugreek seed extracts is shown in Table 3 indicates that ethanol extract has shown higher free radical scavenging activity (62.97±1.56%) as compare to methanol (48.70±1.57%), acetone (20.56±0.90%), dichloromethane (16.16±0.81%) and water (13.93±1.16%). These results are in close agreement with findings of previous researchers. Juhaimi *et al*. (2012) determined the antioxidant potential of seven date seed varieties by using DPPH method and concluded that antioxidants strength ranged between 78.03-79.94mg/mL. For good understanding of fenugreek antioxidant strength, various extracts were prepared in a research planned by Bukhari *et al*. (2008) in terms of remaining amount of DPPH in each solvent extract and these were 21.97%, 25.77%, 18.38%, 24.04%, 12.42% and10.88% in samples

extracted with ethyl acetate, hexane, acetone, dichloromethane, methanol and ethanol, respectively.

Flavonoids

Flavonoids are a diverse group of polyphenolic components with exceptional strength to act as free radical scavenger with many health benefits. Means for total flavonoids of date seed extracts (Table 4) for five solvents; 50% ethanol, 50% methanol, 50% acetone 50% dichloromethane and aqueous are 652.27±31.13, 560.80±15.63, 332.29±30.58, 168.71±9.17 and 119.24±8.49mg/100g, respectively. Means shown in Table 4 for total flavonoids of fenugreek seed extracts are 329.71±18.95, 158.73±6.44, 126.15±8.97, 89.26±10.53 and 76.17±2.3949mg/100g for 50% ethanol, 50% methanol, 50% acetone, 50% dichloromethane and for aqueous extracts, respectively. Mistrello *et al*. (2014) determined the potential of flavonoid contents in different date seed varieties. According to the research, flavonoids accounted for the majority of the total phenolics, varied between 1932mg CAE /100 g of date seed powder on dry weight basis in the Deglet Nour and 1271mg CAE /100 g of dry seed powder on dry weight basis in Khouat Allig variety. According to Bukhari *et al*. (2008) solvent plays the key role in determining the extraction efficiency of the plant components. Owing to high polarity, methanol and ethanol are highly suitable for extraction purpose and they possess high yield of phenolic compounds as compared to the other solvents. Different solvents such as methanol, ethanol, hexane, ethyl acetate and acetone were used for extraction and they have showed flavonoid content in the range of 607±3.6, 653±4.3, 208±4.2, 251±3.3 and 416±2.7 (QE µg/g) of fenugreek.

Phenolic acids in date seeds extracts

Among the phenolic compounds of date seeds; gallic, chlorogenic, coumeric, ferulic, sinapic, vanillic, syringic, caffeic and cinamic acids were detected. The concentrations of individual phenolic acid in date seeds are presented in Table 5. Five types of solvent's extracts were used for HPLC analysis, namely ethanol, methanol, acetone, dichloromethane and aqueous. Results of the present study revealed that ethanol extract was the most efficient extract for HPLC analysis. More phenolic acids were detected in ethanol extract among others. The peak values for these phenolic acids of various extracts are shown in Figures 1-5.

Gallic acid was a common acid which was found in all the extracts. Acetone extract showed the highest peak of gallic acid (3.86µg/g). Chlorogenic acid was detected in methanol, acetone and aqueous extract and acetone extract showed the highest peak containing 17.62µg/g followed by aqueous and methanol which were found in the concentrations of 8.65µg/g and 1.51ug/g, respectively. Coumeric acid was detected in ethanol, acetone and aqueous extracts. The highest peak was shown by aqueous extract (4.21µg/g) followed by acetone and ethanol containing 3.51µg/g and 3.38µg/g, respectively. Ferulic acid was the main phenolic acid which was detected in ethanol extract. It had the highest concentration of 24.19µg/g followed by ferulic and sinapic acids in ethanol extract which gave the concentrations of 7.55µg/g and 7.31µg/g, respectively. The concentrations of vanillic and syringic acids were also found in sufficient amounts while gallic and coumeric acids were found in low concentrations in ethanol extract. Chlorogenic, caffeic and cinamic acids were not found in ethanol extract. Ferulic acid was detected in methanol extract in a concentration of 15.48µg/g. Sinapic acid was detected in ethanol, methanol and in dichloromethane extracts, whereas the ethanol extract showed the highest peak (7.55µg/g). Vanillic acid was detected in all the extracts of date seeds while the highest amount was detected in dichloromethane extract (20.51µg/g) and the lowest in methanol extract (2.41µg/g). Syringic acid was only detected in ethanol extract (3.32µg/g). Caffeic and cinammic acids were detected only in acetone extract. The results of present study are found similar with the findings of previous investigators. Al-Farsi and Lee (2008) worked on the phenolic compounds of date seeds and reported that the nine major phenolic acids are present in date seeds, out of which four consist of benzoic acid derivatives (gallic acid, p-hydroxybenzoic, protocatechuic acid, vanillic acid) and five were derivatives of cinnamic acid (caffeic acid, ferulic acid, *p*-coumaric acid, m-coumaric and 0-coumaric acid).

Phenolic acids in fenugreek seeds extracts

Among the phenolic compounds of fenugreek seeds; gallic, chlorogenic, coumeric, sinapic, vanillic, syringic, caffeic, cinamic and benzoic acids were detected. The concentrations of individual phenolic acid in fenugreek seeds extract are presented in Table 6. Five types of solvents were used for HPLC analysis, namely ethanol, methanol, acetone,

Table 1. Mean value for yield estimation of date and fenugreek seeds extracts with different solvents

Solvents	Date seed (%)	Fenugreek seed (%)
Ethanol	36.83± 0.63	27.68±0.96
Methanol	36.19± 0.09	27.03±0.13
Acetone	18.72± 0.22	16.36±1.26
Dichloromethane	14.24± 0.35	11.76±0.57
Aqueous	10.53± 1.63	8.93±0.31

Table 2. Mean value for TPC of date and fenugreek seeds extract with different solvents

Solvents	Date seed (mgGAE/100g)	Fenugreek seed (mgGAE/100g)
Ethanol	2404.80±167.64	959.05±32.68
Methanol	1671.00±58.06	739.12±22.01
Acetone	900.43± 54.63	583.96±37.26[c]
Dichloromethane	765.33±38.67	295.89±20.19
Aqueous	718.10±15.67	125.05±17.56

Table 3. Mean value for DPPH of date and fenugreek seeds extract with different solvents (%)

Solvents	Date seed	Fenugreek seed
Ethanol	76.15±2.19	62.97±1.56
Methanol	74.20±1.82	48.70±1.57
Acetone	63.12±2.80	20.56±0.90
Dichloromethane	48.94±2.45	16.16±0.81
Aqueous	42.10±1.73	13.93±1.16

Table 4. Mean value for TFC of date and fenugreek seeds extract with different solvents (mg/100g)

Solvents	**Date seed**	**Fenugreek seed**
Ethanol	652.27±31.13	329.71±18.95
Methanol	560.80±15.63	158.73±6.44
Acetone	332.29±30.58	126.15±8.97
Dichloromethane	168.71±9.17	89.26±10.53
Aqueous	119.24±8.49	76.17±2.39

dichloromethane and aqueous. Results of the present study revealed that methanol extract was the most efficient extract. More phenolic acids were detected in methanol extract among others. The peak values for these phenolic acids of various extracts are shown in Figures 6-10.

Gallic acid was a common acid that was detected in all the extracts. Gallic acid was the main phenolic acid which was detected in methanol extract. It had a concentration of 65.26µg/g. Sinapic and benzoic acids were determined as the second and third which contained the highest quantities in methanol extract (16.13µg/g and 16.21µg/g, respectively). The concentrations of vanillic and coumeric and caffeic acids were also found in sufficient amounts in methanol extract. Chlorogenic, syringic and cinamic acids were not found in methanol extract. Aqueous extract showed the highest peak of gallic acid (87.26µg/g). Chlorogenic acid was only detected in dichloromethane extract (16.45µg/g). Coumeric acid was detected in all the extracts. The highest concentration was found in acetone extract (31.14µg/g) followed by methanol, aqueous, dichloromethane and ethanol extracts as they had the concentrations of 6.48, 2.83, 2.76 and 1.45µg/g, respectively. Sinapic acid was detected in acetone and methanol extracts, whereas the acetone extract showed the highest peak (27.97µg/g). Vanillic acid was only detected in methanol extract (10.85µg/g). Syringic acid was only detected in dichloromethane extract (4.84µg/g). Caffeic acid was detected in ethanol, acetone and dichloromethane extracts whereas cinammic acid was only detected in aqueous extract (13.45µg/g). Benzoic acid had showed its presence in methanol and aqueous extracts in the tune of 16.21 and 11.44µg/g, respectively.

Antibacterial activity of date and fenugreek seed extracts against *Escherichia coli*

Mean values (Table 7) illustrated that zone of inhibition of date seeds extract with 50% ethanol shows higher value of inhibition zone (24±1 mm), while inhibition zone for 50% methanol, 50% acetone, 50% dichloromethane and aqueous extract were 18±1mm, 13±1mm, 10±1mm and 6±1mm, respectively. Means for disc diffusion of fenugreek seed extracts (Table 7) for five solvents; 50% ethanol, 50% methanol, 50% acetone, 50% dichloromethane and aqueous were 24±1, 19±0.5, 12±1, 7±1.52 and 4±1mm, respectively. These results showed variable zones of inhibition which might be

Table 5. Concentration of phenolic acids (µg/g) in date seeds extract through HPLC with different solvents

Phenolic acids	Ethanol	Methanol	Acetone	Dichloromethane	Aqueous
Gallic acid	1.49	0.38	3.86	1.36	1.26
Chlorogenic acid	-	1.51	17.62	-	8.65
p-coumeric acid	3.38	-	3.51	-	4.21
Ferulic acid	24.19	15.48	-	-	-
Sinapic acid	7.55	0.31	-	7.36	-
Vanillic acid	7.31	2.41	13.72	20.15	6.14
Syringic acid	3.32	-	-	-	-
Caffeic acid	-	-	5.16	-	-
Cinamic acid	-	-	68.88	-	-

Table 6. Concentration of individual phenolic acids (µg/g) in fenugreek seeds extract through HPLC with different solvents

Phenolic acids	Ethanol	Methanol	Acetone	Dichloromethane	Aqueous
Gallic acid	28.98	65.62	123.32	11.73	87.26
Chlorogenic acid	-	-	-	16.45	-
p-coumeric acid	1.45	6.48	31.14	2.76	2.83
Sinapic acid	-	16.13	27.97	-	-
Vanillic acid	-	10.85	-	-	-
Syringic acid	-	-	-	4.84	-
Caffeic acid	-	5.01	19.64	15.64	-
Cinamic acid	-	-	-	-	13.45
Benzoic acid	-	16.21	-	-	11.44

Table 7. Mean value for disc diffusion (mm) of date and fenugreek seeds extract with different solvents

Solvents	Date seed	Fenugreek seed
Control	35±1	33±1
Ethanol	24±1	24±1
Methanol	18±1	19±0.5
Acetone	13±1	12±1
Dichloromethane	10±1	7±1.52
Aqueous	6±1	4±1

Table 8. Mean values for MIC (mg/mL) of date and fenugreek seeds extract with different solvents

Solvents	Date seed	Fenugreek seed
Ethanol	1.66±0.19	0.94±0.10
Methanol	3.51±0.51	1.51±0.23
Acetone	7.51±0.52	2.36±0.23
Dichloromethane	1.30±0.65	3.72±0.25
Aqueous	13.99±0.32	8.51±0.45

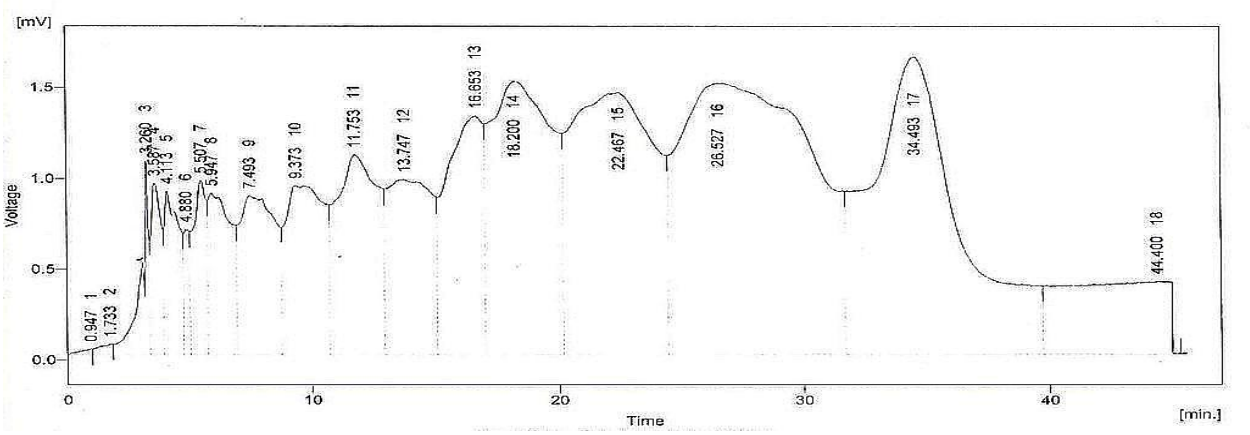

Fig 1. Peak value of phenolic acids (µg/g) of date seeds for ethanol extract

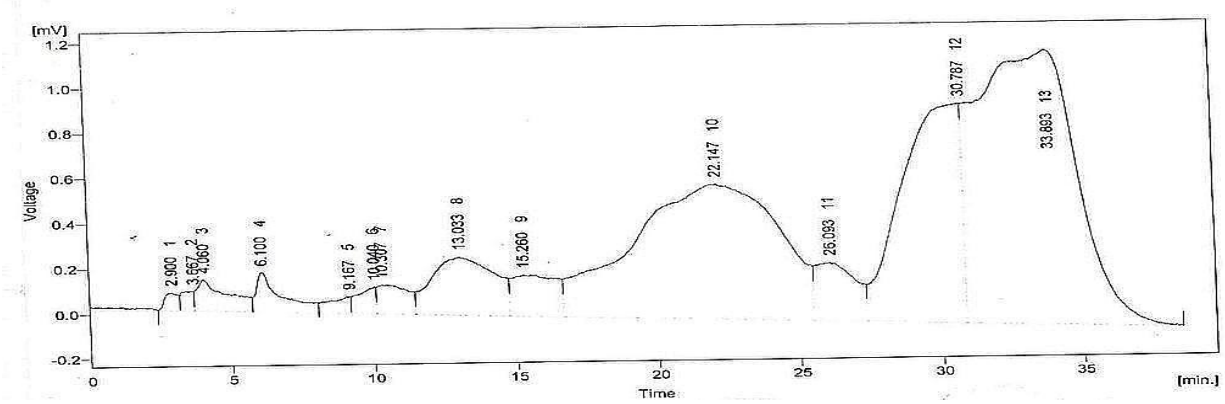

Fig 2. Peak value of phenolic acids (µg/g) of date seeds for methanol extract

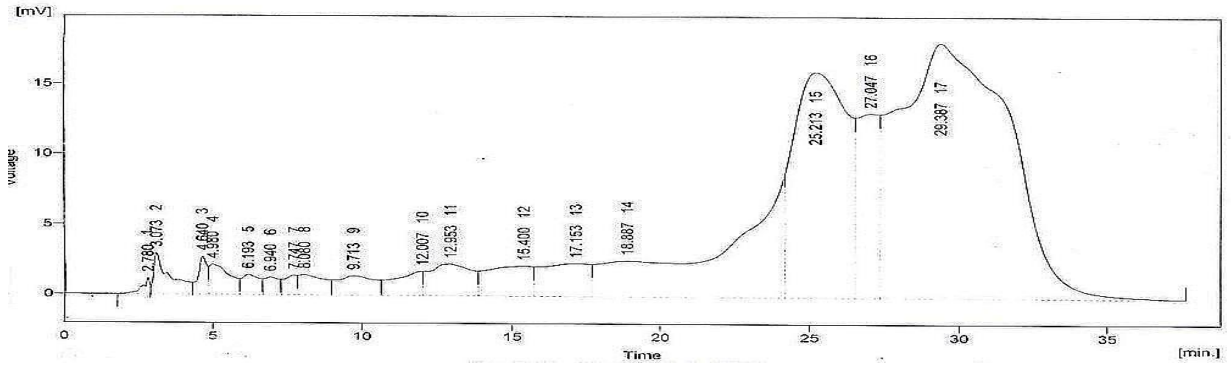

Fig 3. Peak value of phenolic acids (µg/g) of date seeds for acetone extract

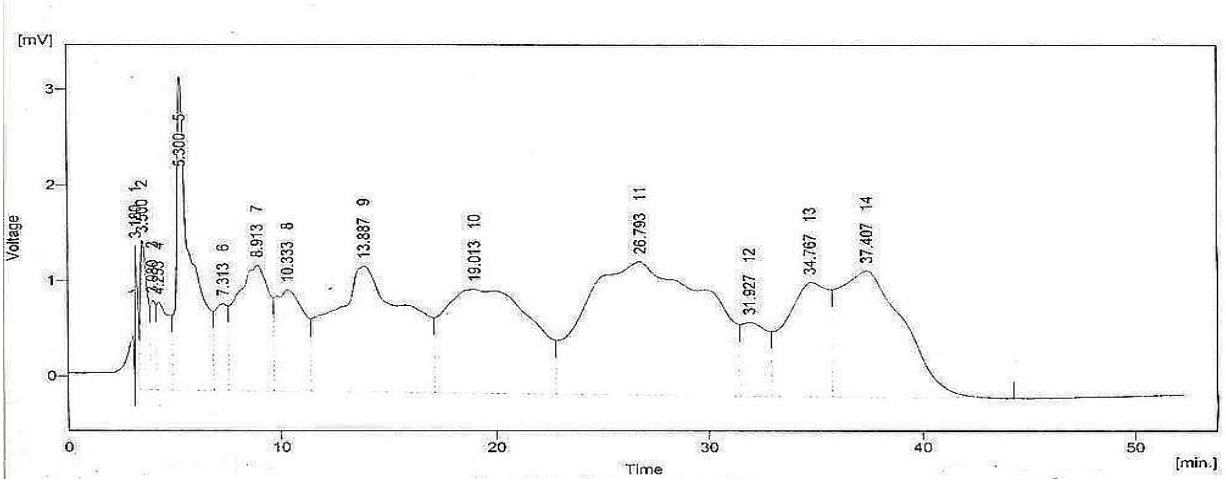

Fig 4. Peak value of phenolic acids (µg/g) of date seeds for dichloromethane extract

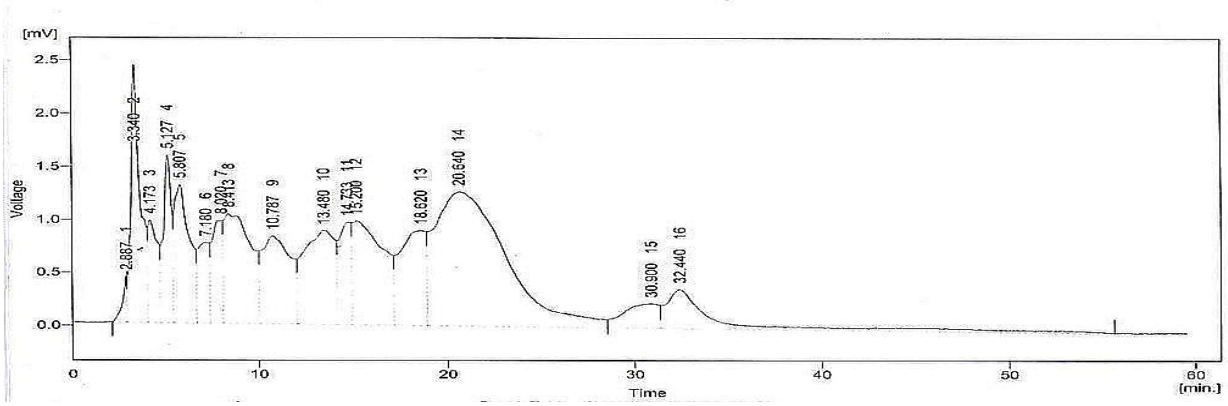

Fig 5. Peak value of phenolic acids (µg/g) of date seeds for aqueous extract

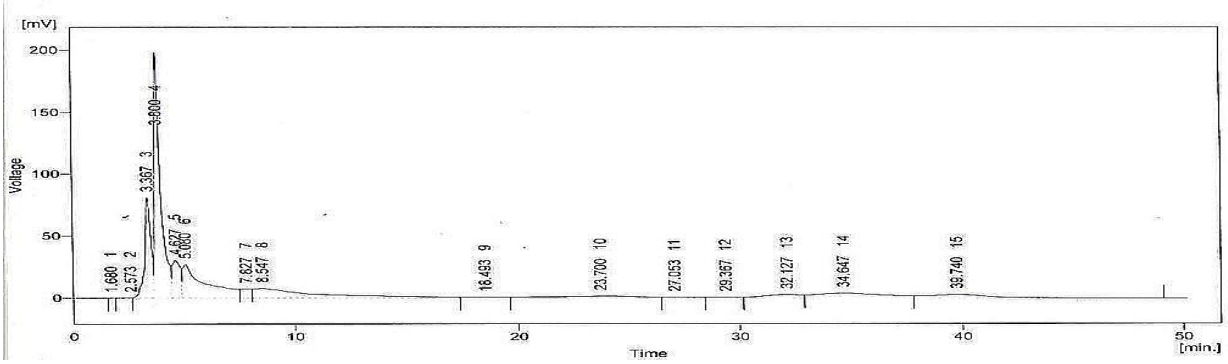

Fig 6. Peak value of phenolic acids (µg/g) of fenugreek seeds for ethanol extract

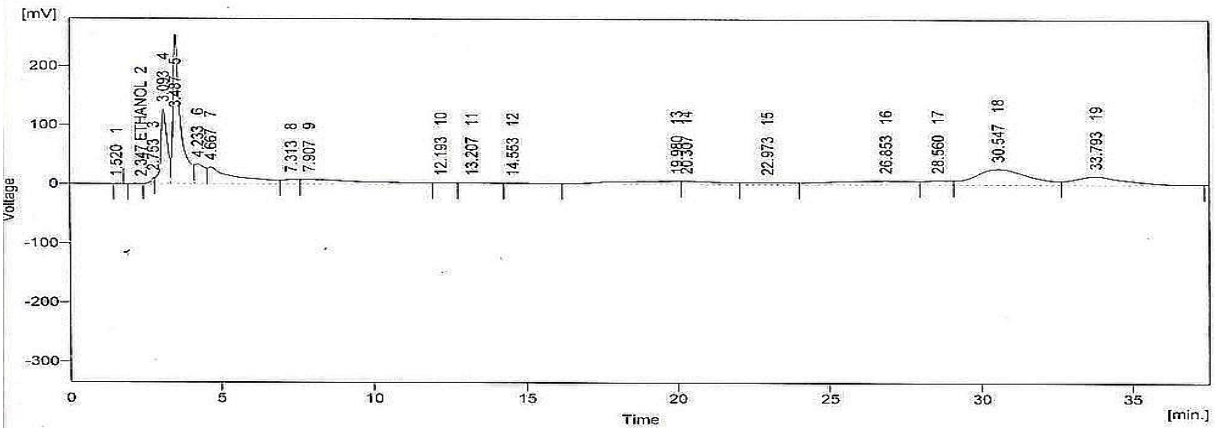

Fig 7. Peak value of phenolic acids (µg/g) of fenugreek seeds for methanol extract

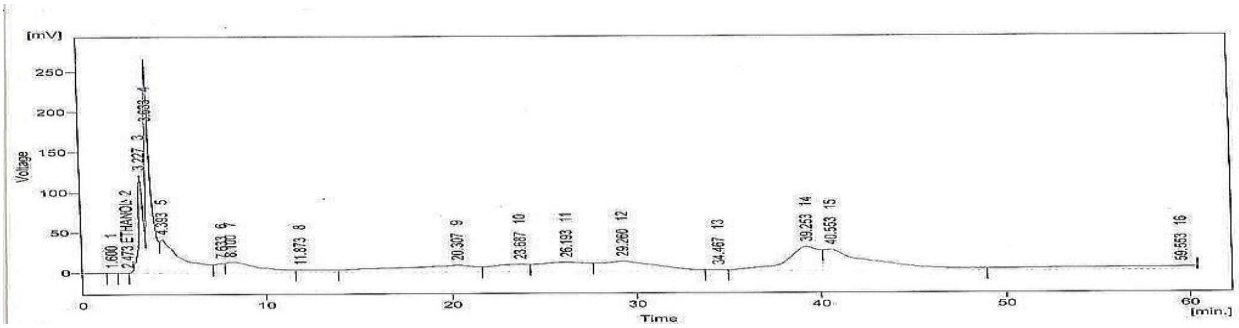

Fig 8. Peak value of phenolic acids (µg/g) of fenugreek seeds for acetone extract

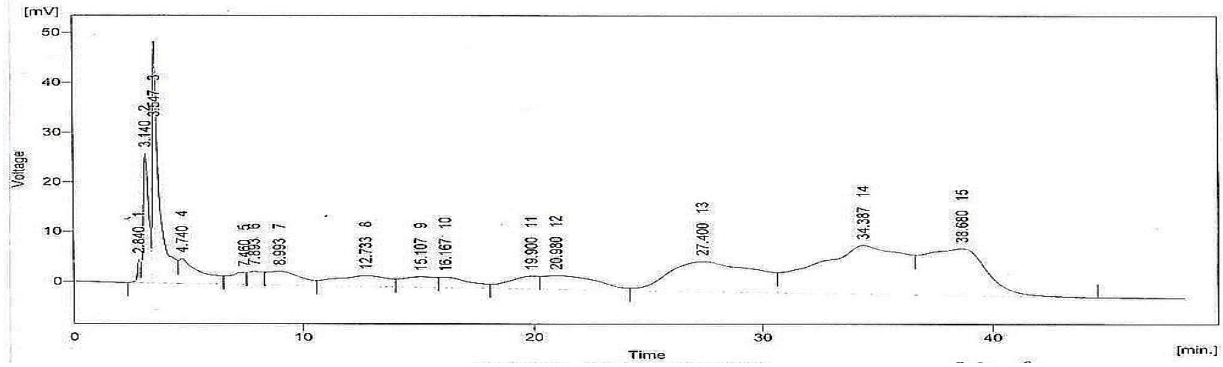

Fig 9. Peak value of phenolic acids (µg/g) of fenugreek seeds for dichloromethane extract

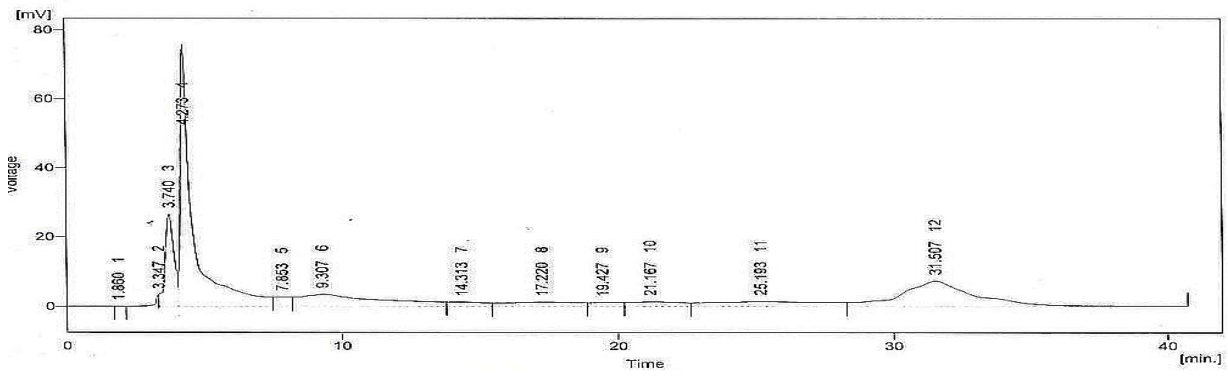

Fig 10. Peak value of phenolic acids (µg/g) of fenugreek seeds for aqueous extract

due to use of different solvents for extraction purpose.

Bhat and Al-Daihan (2016) worked on the antibacterial activity of date fruits and seeds of five different varieties and concluded that date fruits showed highest inhibition zone ranges from 10-25mm as compared to the seeds of date fruit which ranges from 10-13mm against *E.coli*. Al-Daihan and Bhat (2012) also reported the antibacterial activity of date fruits, leaves, seeds and bark by using three solvents like methanol, acetone and aqueous. According to their findings, date seed extracts showed inhibition zones against *E.coli* were 10±0.00, 11±0.89 and 11.60±0.88mm for aqueous, methanol and acetone extracts, respectively. Chitra *et al*. (2014) studied the antimicrobial activity of fenugreek seeds and they concluded that fenugreek seeds showed maximum inhibition zone ranged between 21-24mm against *E.coli*. Al-wihibi and Soliman (2014) determined the inhibition zone of fenugreek seed extract prepared by using five different slovents; water, acetone, chloroform, ethanol and methanol that showed inhibition zones of 5.2, 18.2, 28.4, 25.2 and 22.6 mm, respectively.

Minimum inhibitory concentration (MIC) for *Escherichia coli*

Mean values for MIC of date seed and fenugreek seed extracts are presented in Table 8 that indicate that MIC values were found changing with changing in the solvents. Ethanol extract of date seed showed the maximum antimicrobial activity (1.66±0.19mg/mL) followed by methanol, acetone, dichloromethane and aqueous extract. Aqueous extract showed the minimum inhibitory concentration of 13.9±0.32mg/mL. MIC of fenugreek seeds also revealed that ethanol extract showed the highest antimicrobial activity (0.94±0.10) among other solvents. These results are found agreed with findings of previous research. Bhat and Al-Daihan (2012) worked on the antimicrobial activity of different date seed varieties. Minimum inhibitory concentration reported by them was recorded between the range of 1.3-3.2 mg/mL. Chitra *et al*. (2014) worked on the fenugreek seeds extract prepared by using acetone. They reported that fenugreek extract showed maximum activity against *Escherichia coli*. According to their results, zone of inhibition of 24mm was obtained for the concentration of 8mg/mL, whereas 22mm and 20mm were obtained for concentrations of 4 mg/mL and 2 mg/mL for *Escherichia coli*, respectively.

CONCLUSION

The present research work demonstrated that the ethanol extract of both date and fenugreek seeds showed higher antioxidant and antibacterial activities against *Escherichia coli* as compare to other solvents like methanol, acetone, dichloromethane and aqueous. Phenolic acids such as gallic, chlorogenic, coumeric, sinapic, vanillic, syringic, caffeic, cinamic and benzoic acids were detected in fenugreek seeds while gallic, chlorogenic, coumeric, ferulic, sinapic, vanillic, syringic, caffeic and cinamic acids were detected in date seeds.

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Single cell proteins: A novel value added food product

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ABSTRACT

Single cell proteins are the biomass (dried cells) produced by microorganisms including yeast, bacteria, algae and fungi. This biomass contains proteins, amino acids, vitamins and lipid content. The microorganisms utilize waste materials, agricultural and industrial waste, feed stocks and secrete natural protein concentrates. A variety of substrates are available for the production of single cell proteins, most common among them is the yeast which produces 250 tons of proteins in 24 h. Single cell proteins can be used as a vital supplement of proteins and regarded as a quantitative approach against malnutrition. Two main reasons for the replacement of single cell proteins by conventional source are its high nucleic acid content and low digestibility. Single cell proteins have a wide field of applications in animal nutrition, in food stuffs and in technological fields. The production process and research and development of single cell proteins are encompassing various fields of science including genetics, microbiology, biotechnology, economics, agriculture, food technology and veterinary sciences.

Keywords: Single cell protein, Bacteria, Sources, Substrate, Biotechnology, value -added product

INTRODUCTION

The term 'single cell protein (SCP)' was first introduced in 1968 at the Massachusetts Institute of Technology (MIT) in a meeting to find the alternate of most commonly used terms i.e. petroprotein and microbial protein. Many microbial species such as bacteria, fungi and algae are involved in the production of single cell protein. Bacteria and fungi are the potential microbes for SCP production. High content of protein and rapid growth are the characteristics that made them an important and useful source. A variety of algal species are cultivated from aquatic medium that are generally used for the production of Single Cell Protein (Aggelopoulos *et al*., 2014; Gao *et al*., 2007; Paraskevopoulou *et al*., 2003).

Technically, cell mass is manufactured by Singe Cell Protein with the corporation of microorganisms and this process is carried out by using available waste as culture medium. Bacteria, fungi and algae are the main sources that produce microbial protein and these can be used as Single Cell Protein. Microbial biomass is produced by using either solid or submerged fermentation process. Once the fermentation is done, the harvesting of biomass is carried out, this can be utilized as a source of protein and is subjected for further processing steps such as cell disruption, purification, washing and protein extraction. Generally there is high yield of protein and production rates, the production control process also become easy. This process makes the Single Cell Protein more attractive source of protein when compare with other animal and plant sources (Anupama and Ravindra, 2000).

Single-cell protein when produced by utilizing waste material it provides economically important protein source that can be used as animal feed or it may also undergo further processing for human consumption. A variety of microbes are involved in the conversion of various industrial wastes and substrates into biomass (Voltolina et al., 2005). Cyanobacterium is studied for the waste water treatment and SCP production, this may also increase the residual value of agro-industry (Jacob-Lopes *et al*., 2006; Queiroz *et al*., 2007; Zepka *et al*., 2008).

Several studies are carried out to investigate the utilization of hemicelluloses and cellulose as suitable substrate in the production of SCP, many other substrates such as carbon are also considered to be useful in the production of SCP (Nasseri *et al*., 2011). Similarly for many instance the hydrolization of raw material was carried out by enzymatic, physical and chemical methods. Various agricultural wastes like cellulose and hemicelluloses from plants, polysaccharides, fibrous proteins like nail, hair, feather and horn, nitrogenous compounds and hydrocarbons can also be utilized to produce Singe Cell Protein (Ashok *et al*., 2000).

Sources of single cell protein

Bacterial sources

Bacteria have short generation time, the cell mass of bacteria multiply within 20min-2hrs and they can grow rapidly, due to these characteristics bacteria are suitable in the production of SCP. They also have the ability to grow on different types of raw material ranging from liquid hydrocarbons such as fractions of petroleum and methane to gases and carbohydrates like sugars and starches (Bamberg, 2000) to wastes of organic nitrogen and petrochemicals which include nitrogen, ethanol and methanol. It is also suggested to add mineral nutrient supplement that help the bacterial culture to fulfill deficiency of nutrients which is required in sufficient concentration for the growth in natural water. Potential phototrophic bacterial strains are recommended for single cell protein production. Some researchers also suggest use of methanotrophic and other bacterial species for single cell protein production. The Methylophilusis generation time almost 2 hrs is useful for animal feed but generally they can produce favorable composition of protein than fungi or yeast. Therefore animal feed can produce a large quantity of single cell protein by using bacteria like *Brevibacterium* (Adedayo *et al*., 2011) *Acinetobacter calcoacenticus, Methylophilus methylitropous, Bacillus megaterium, Acromobacter delvaevate*, *Bacillus subtilis* (Gomashe *et al*., 2014), *Aeromonas hydrophilla, Cellulomonas species, Methylomonas methylotrophus*, *Lactobacillus species*, (Piper, 2004), *Thermomonospora fusca, Flavobacterium species, Pseudomonas fluorescens, Rhodopseudomonas capsulate,* (Dhanasekaran *et al*., 2011).

Algae

Since ancient times, *Spirulina* was cultivated by people in africa near lakechad and in mexico near Texcoco. They used it as a food after drying it. *Spirulina* is the most widely used algae so much that even astronauts during their space travel take it to space. Similarly, biomass obtained from *Senedessmus* and *Chlorella* has been harvested and used as source of food in many parts of world. Alga is used as a food in many different ways and its advantages include high content of protein, simple cultivation, rapid growth and beneficial use of solar energy. The algae *Spirulina* has been considered for use as a supplementary protein (Raja *et al*., 2008). It is a green algae that excite the free radical in enzyme system and exhibits antioxidant activity. Healthy diet containing nutraceuticals and *Spirulina maxima* have the ability to protect progenitor/stem cells. This can also prevent the development of fatty liver due to carbon tetrachloride (CCl4). It is concluded that the use of *Spirulina* should be encouraged in patients suffering from malnutrition, immune suppression, hepatic, neural compromise and etc. In a study, the production of SCP from five different strains of *Chlorella* (M150, M122, M121, M109 and M138) was isolated from a variety of habitats and also studied the effects of eight environmental factors (Mahasneh, 2005). Although, there is a need of further investigations on the antiviral effects of this alga and its clinical implications.

Fungal sources

As a source of protein rich food many fungal species are used (Bhalla *et al*, 2007). Many other filamentous species are also used as source of single cell protein. In 1973 during second international conference convened held at MIT, it was reported that *Actinomycetes* and filamentous fungi produced protein from various substrates. For the period of the world war II, trials were made to utilize the cultures of *Rhizopus* and *Fusarium* (Yousuf, 2012) as a source of protein food are grown in fermentation. The inoculums of *Rhizopus arrhizus* (Anupama and Ravindra, 2000*)* or *Aspergillus oryzae* were selected due to their nontoxic nature. On complex organic compounds saprophytic fungi are grow and convert them into simple structures. High amount of fungal biomass is produced as a result of growth. Mycelia yield vary greatly which depends upon substrates and organisms.

There are some species of moulds such as *Aspergillus niger* (Yabaya and Ado, 2008), *A. fumigates*, *Fusarium graminearum* which are very dangerous for human, that the reason, such fungi, must not be used or before recommending to use as SCP toxicological evaluations should be done. Very recently, SCP technology is using fungal species for bioconversion of lignocellulosic wastes (Lenihan *et al*, 2010). The type of filamentous fungi that have been used are *Fusarium graminearum, Chaetomium celluloliticum* (Zubi, 2005), *Aspergillus fumigates, A.oryzae, A. niger, Cephalosporium cichorniae, Rhizopuschinensis, Scytalidum aciduphlium, Penicillium cyclopium, , Tricoderma alba Paecilomyces varioti and Tricoderma viridae* (Jaganmohan *et al*., 2013).

Yeast

Yeast single-cell protein (SCP) is a high nutrient feed substitute (Burgents *et al*, 2004). Among these, most popular are yeast species *Candida* (Bozakouk, 2002), *Hansenula*, *Pitchia*, *Saccharomyces* and *Torulopsis*. The production of SCP by using *Saccharomyces cerevisiae* grown on various fruit waste (Tanveer, 2010). The usual oily yeasts genera contain *Yarrowia, Candida, Cryptococcus, Rhodotorula, Rhodosporidium, Lipomyces and Trichosporon*.

Orange peels and cucumber were evaluated for the production of SCP by using *Saccharomyces cerevisiae* by submerged fermentation (Sengupta *et al*., 2006).

Production of single cell protein

Precisely, SCP is manufacture of the cell mass by using different microorganisms that culturing on profusely available industrial and agriculture wastes. The production of microbial biomass is done either by a solid state fermentation submerged process. Biomass is harvested after fermentation and it may be subjected towards different downstream processing steps such as washing, protein extraction, cell disruption and purification.

Table 1. Substrate for the production of single cell protein

Production from fungi	
Organisms	**Substrate**
Sporotrichum pulverulentum	Maize and Cotton stalk
Candida tropicalis ceppo 571	Sulfite waste liquor
Aspergillus niger AS 101	Corn cobs
Chrysonilia sitophilia	Lignin
Chaetomium cellulolyticum	Cellulosic wastes
Marine yeast	Pawn shell wastes
Fusarium graminearum	Starch hydrolysates
Paecilomyces variolii	Sulfite liquor
Mixed cultures of yeasts	Dairy wastes
Pichia pastoris	Methanol
Penicillium camemberti	Citus fruit peel
Penicillium cyclopium	Whey
Trichoderma album	Not disclosed
Schwanniomyces occidentalis	Starch
Scytalidium acidophilum	Waste paper
Saccharomyces cereviceae	Molasses, Stillage
Yeast	Plant origin liquid waste
White rot fungi	Sugarcane bagasse
Trichoderma reesei & *Kluyveromyces marxianus*	Beet-pulp

Production from bacteria	
Organisms	**Substrate**
Bacteria of *Methylococcaceae*	C1 compounds
Brevibacterium spp.	C1–C4 compounds
Cellulomonas spp.	Agricultural wastes
Pseudomonas fluorescens	Manure, Animal wastes
Methylophilus methanotrophus	Methanol
Methanomonas methanica	Methane
Streptomyces spp	Methanol
Rhodopseudomonas gelatinosus	Wheat bran
Different species of bacteria	Fruit processing wastes

Production from algae	
Organisms	**Substrate**
Chlorella salina CU-1(28)	Saline sewage effluent
Caulerpa rocemosa	Carbon dioxide +1 sunlight
Chlorella spp.	Carbon dioxide
Dunaliella	Carbon dioxide and sunlight
Chlorella & Diatoms	Carbon dioxide and sunlight
Laminaria	Carbon dioxide and sunlight
Porphyra	Carbon dioxide and sunlight
Sargassum	Carbon dioxide and sunlight
Spirulina maxima	Carbon dioxide and sunlight
Spirulina spp.	Carbon dioxide

(Anupama and Ravindra, 2000)

Single cells proteins production by fermentation

The fermentation process requires a pure culture of specified microorganisms which was grown on suitable raw materials and then it is separated by screening in correct physiological state. This process contains a fermenter for the process to be carried out. A fermenter is provided with all the equipment needed to run the process smoothly. It is included a thermostat for the temperature regulation, pH detector for the measurement of pH, aerator for continuous supply of oxygen and a stirrer. Culture medium is placed in fermenter and the process is carried out leading to the cell separation and the supernatant of the cell is collected. The product is then obtained by protein extraction and purification and the effluent is treated (Ravindra, 2000; Nasseri *et al*., 2011).

a. Submerged fermentation

Submerged fermentation contains the liquid form substrate is used which provides all the nutrients required by the microorganism for growth. Operational conditions are applied continuously during fermentation process and the product is harvested after regular intervals. The harvested biomass is filtered and the centrifuged. Single cell proteins are then obtained after the process of drying. Submerged fermentation is operated at a high cost and has more capital investment (Nasseri *et al*., 2011).

b. Semisolid fermentation

The substrates preparations in solid state fermentations is less clear and are used more in the solid form rather than liquid. The process of cultivation is carried out by stirring of multiphase. The oxygen is transferred to the microorganisms in the form of bubbles through liquid phase. This liquid phase also regulates the temperature of the process. A special bioreactor called u-loop fermenter is used in solid state fermentation. Process is carried out by sterilization of the fermenter and growth medium, growth medium with suitable carbon source, production of specific microorganisms, harvesting of product biomass, its processing and purification (Nasseri *et al*., 2011)

c. Solid state fermentation

Solid state fermentation is being extensively used for the production of solid cell proteins, enzymes, organic acids, pigments and flavor. Solid state fermentation is carried out in solid substrates with no free water and does not require pre arrangements of preparation of growth media. Fungi show good growth in low water activity and yields high product biomass as compared to submerged fermentation. Solid state fermentation involves efficient utilization of waste which act as solid substrate and produce commercial viable cells. The process mainly involves seeding of the rice or bran substrate with microbial cells. Then the substrate is left for several days in the form of flat beds. Then harvesting of cells, further processing and finally drying of the cells is carried out (Nasseri *et al*., 2011).

Nutritional significance of single cell proteins

Nutritional assessment of single cell proteins includes the content and composition of nutrients, amino acids and vitamins. Hence it can be used as an alternative food by the living microorganisms. Moreover, the nutritional benefits depend on its digestibility and allergic reactions when used for human consumption. Single cell proteins includes not only proteins but also characterized as an important source of essential amino acids, carbohydrates, lipids and nucleic acids. The composition of single cell proteins depends on the substrate used for the production and suitable microorganism for high yield (Zepka *et al*., 2010; Bogdahn, 2015).

Yeast is the most suitable microorganism for single cell protein production and produces the products with 50-55% protein. Yeasts are used as animal feed and are characterized by high contents of amino acids, B-group vitamins and lysine. Yeasts contain fewer amounts of methionine and cysteine and hence limit their use as single source of proteins. Yeast contains 2-6% fats and 6-12% nucleic acids. *Pitchia, Candida, Hansenula, Saccharomyces and Torulopsis* are the important species used in the production of single cell proteins. *Saccharomyces cerevisiae* is the probiotic strain of yeast utilizes fruit waste as substrates (Ferreira *et al*., 2010; Gao *et al*., 2007).

A number of fungal species are being consumed as rich protein sources. It was reported that *Fusarium* and *Rhizopus* are tried to be used as alternative protein sources during World War II (Yousufi, 2012). Algae contain large amounts of proteins, vitamins and fats. Alga is used as food for its high protein content. An Alga *Spirulina* is blue green algae is used as supplementary proteins and have strong antioxidant activity (Ravindra, 2000).

Single cell proteins; a key to curtail protein malnutrition

Single cell proteins are playing a major role in providing alternative source of proteins, hence reducing the problems of malnutrition. A single cell protein not only provides proteins but also contains carbohydrates, vitamins and minerals. Microorganisms including yeast, bacteria and fungi produce single cell proteins by utilizing the industrial

wastes and different substrates that meets the energy requirements for proteins.

Table 2. Vitamins contained by different species of algae

Species of algae	Vitamins
Macrocystis	Vitamins A and E
Nitzschia	Vitamin A
Ulva, Enteromorpho, Laminaria, *Alaria valida* and *Porphyra*.	Vitamin B
Ulva, Enteromorpho, Alaria valida	Vitamin C

(Ravindra, 2000)

Cyanobacteria are also utilized for the production of single cell proteins. These single cell proteins are characterized by low content of nucleic acids and high amount of proteins. A cyanobacterium grows rapidly and produces good quality of proteins. In the treatment of waste waters, a cyanobacterium called Aphanothece has been studied to utilize the nitrogen and organic matter present in effluent of parboiled rice. It yields higher protein content when compared to the routine foods like eggs, wheat meal and meat (Jacob-Lopes *et al*., 2006; Queiroz *et al*., 2007; Zepka *et al*., 2010)

Toxic compounds produced by single cell proteins

The name, physiological characteristics and composition of the raw materials used for the production of single cell proteins are the major sources of hazards and toxicity. The final product is tested and investigated for any toxic compound present. This testing includes short term acute toxicity to long term extensive toxicity. The toxic compounds can cause allergy and various gastrointestinal problems.

a. Nucleic acids

Nucleic acids are the byproducts obtained in the production of single cell proteins. The degradation of nucleic acid leads to the formation of uric acid. Due to the absence of an enzyme called urease, the uric acid accumulates in the human body. Hence, nucleic acid must be reduced to the amounts which are not harmful for human health. An amount more than 2g/day is not good for human body and can lead to formation of stones in kidney. Nucleic acid content is reduced in number of ways. RNA is the major constituent in the formation of nucleic acids. The RNAase enzyme is activated usually by heat treatments for its reduction. The RNA content of yeast also depends on the conditions of growth culture. Hence by optimizing these conditions its content could be reduced. Other treatments include hydrolysis of nucleic acid by alkali and chemical extraction (Ravindra, 2000).

b. Toxins

Toxicity is also caused by the production of toxins by the microorganisms. These toxins act as contaminants in the food product. Yeast, algae, bacteria produce toxins during their growth period as secondary metabolites. These toxins are tested after the production of single cell proteins products. Test and trial are made on the live animals to test whether it is suitable for consumption as animal feed or as food product by humans.

c. Mycotoxins

The elimination of mycotoxins has becoming a biotechnological problem because its minute quantities in food are causing serious health problems. The presence of mycotoxins is generally assessed by thin layer chromatography or high performance liquid chromatography (HPLC) procedures. Among mycotoxins, alfatoxins, ochratoxins, Trichothecenes are primarily understood in the history. Alfatoxins are produced by *A. parasiticus*, *P. cizaetrinum*, *A. flavus* and *A. oryzae*. Alfatoxins must be eliminated or reduced by method of ammoniation which reduces the level of alfatoxins by 99%. A report showed that a number of turkeys died after eating the food contaminated with alfatoxins. Sometimes, the non-toxic strains added in the growth medium produces toxins in the product of single cell proteins, so the products must be checked by the performance of analysis and testing procedures (Ravindra, 2000).

Another method employed for the elimination of mycotoxins is the application of molecular biology techniques. These techniques involve some procedures of genetic engineering for the alteration of specific gene that leads to the production of mycotoxins.

d. Bacterial toxins

Two types of bacterial strains are produced: endotoxins and exotoxins. Exotoxins are produced by gram positive bacteria in its surrounding medium when cultured in a media. Even Nano gram levels are dangerous for the laboratory animals and can cause death. The exotoxins can produce different types of lesions in the body.

Endotoxins are the toxins produces by gram negative bacteria upon lysis. These are the parts of cell walls in gram negative bacteria. Its amount higher then

exotoxins are fatal and lower amounts causes fever in the body. Endotoxins are difficult to control because these are integral cellular components. Techniques of genetic engineering are applied for the elimination of the gene that produces toxins (Ravindra, 2000).

Limitations for single cell proteins usage

Single cell protein technology has numerous health benefits but apart from that, single cell proteins cannot be adopted currently due to its numerous disadvantages. The nucleic acids produced as a byproduct in the production of single cell proteins constitute 6-10%. These nucleic acids increase the quantity of uric acid in the body. This leads to the formation of stones in the kidney and other organs. The single cell proteins obtained from algae, bacteria and fungi contains high amount of nucleic acids. The cell wall of microorganisms contains indigestible component called cellulose which is not digested by the human. Hence the cell components of algae, fungi which must be removed by before the consumption of single cell proteins. Single cell proteins when used for animal feed, the removal of cell wall is not necessary as animals contain cellulose enzyme and various protozoa in the rumen which digest cellulose. Presence of unwanted flavors and odors is also a major problem. The intake of foreign proteins also leads to the problems of indigestion, skin allergies, nausea, vomiting and various disorders. Algae produce single cell proteins with high amount of chlorophyll that is not suitable for the consumption of humans (Zepka *et al*., 2010).

The popularity of single cell proteins on global scale is reduced due to its high content of ribonucleic acids, minimum cell recovery and greater risk of contamination of the product during its production. The contamination must be removed or minimized by following correct procedures and preparations. Selection of productive species of algae and proper climatic conditions are important aspects in elimination of contamination. Certain species of fungi produces toxins mainly alfatoxins and mycotoxins during the production of single cell proteins. The removal of these toxins is a crucial step in single cell protein production because its presence leads to various detrimental health disorders. These toxins can produce allergic reactions, stomach disorders and liver cancer, sometimes leads to death. The production of single cell proteins by bacteria is limited. It contains small size of cells, hence leads to high cost of production. A psychological issue also arises in the utilization of bacteria as a food product (Ravindra, 2000).

Safety concerns

The safety of the consumers is very much concerned in the utilization of single cell proteins. The contamination present in the products of single cell proteins may be carcinogenic and produces cancer in the body. The foreign proteins may cause digestion problems leading to nausea, vomiting and allergies. The final product after production must be made contamination free and purified. Single cell proteins consumptions not affect the immune system of the humans. This can be checked by test for lymphocyte transformation and foot-pad swelling assay. Immunogenicity of single cell proteins produced by bacteria was checked and found to have no effect on the immune system of rats. Single cell proteins also contain traces of heavy metals as contaminants. These heavy metals even small quantities produce mutations in the body. The safety evaluation of SCP products was based upon the guidelines of US-FDA and US Protein Evaluation Group. Proper sanitation and control measures must be applied for safe production of single cell proteins (Ravindra, 2000).

CONCLUSION

With the increasing awareness of safe and healthy food among the people, consumers are moving towards alternative food supplements to cure nutritional deficiencies. Single cell proteins can combat the problems of protein malnutrition by providing an alternative protein source. Single cell proteins are still not used on global scale as it contains certain toxic elements and contaminants which are reduced to minimum level by applying a number of techniques. A single cell protein contains proteins, amino acids, fats, lipids and nucleic acids. The final packaging of the single cell proteins must provide complete microbiological information of specie, strain contained. Complete analysis and tests of the SCP products produced for the consumption of the human must be performed. Future prospects of single cell proteins involve the utilization of genetically improved species for high yield and production.

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Utilization of pomegranate peel extracts to enhance the stability of sunflower oil

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ABSTRACT

Pomegranate (*Punica granatum* L.) peel possess greater antioxidant potential as well as total phenolics especially extracted by ethanol (3.06 and 82.52) and methanol (3.56 and 83.16), can be used to inhibit lipid oxidation in oil. Pomegranate peel extract (500,750 and 1000 ppm/L) can be used as antioxidant source in the oil to replace commercial synthetic antioxidants. The peel extract addition showed positive effect on the enhancing quality of the oil as evaluated by maximum reduction in free fatty acids (0.2599±0.11%), iodine value (98.06±3.73%), saponification value (192.50±0.56mgKOH/g), peroxide value (2.067±0.44meq/kg) and TBARS (0.1203±0.02) compared to control (0.4667±0.10%, 112.99±3.81%, 194.58±0.88mgKOH/g, 17.648±1.04meq/kg and 0.1819±0.05) at highest level of peel extract (1000 ppm/L). The results of storage study revealed minimum deteriorative changes in sunflower oil blended with peel extract. It is concluded from results that pomegranate peel extract is good source of antioxidant and a potential non-synthetic source of antioxidants for oil stability.

Key Words: Pomegranate, Peel, Extract, Oxidation, Sunflower oil, Oil stability.

INTRODUCTION

Sunflower oil is one of the biggest source of edible oil in Pakistan. The fibrous by-product of oil extraction, which is an inexpensive source of high quality protein, is frequently utilized in animal nutrition (Nagraj, 1997). Composition of the sunflower showed that its seeds are high in protein (20%), potassium (710 mg/100 g), magnesium (390 mg/100 g), oleic (14-40% of total PUFA) and linoleic acids (48-74% of total PUFA) (FSAIFR, 2002). Non-synthethic antioxidants are more preferred over synthetic antioxidants because of general recognition that these are the safe source and do not pose any harm to consumer's health. On the other hand, there are some limiting factors such as the economic cost of extraction is very high and the quantitative potential of the source is also very important (Naz *et al*., 2004)

Fruits and peels of pomegranate (*Punica granatum* L.) have been used in herbal remedies in many countries of the world. Commonly pomegranate peels and its extracts have been used in medicine (Reddy *et al*., 2007; Shan *et al*., 2007). Pomegranate fruit pericarp (peels) is rich in tannins which are high molecular weight phenolic compounds having remarkable antimicrobial activity (Voravuthikunchai *et al*., 2004). The utilization of pomegranate fruits and peel in food processing take great attention due to its potential health benefits. Pomegranate rind powder is extracted using water (Naveena *et al*., 2008). Ethanol, methanol, acetone, and chloroform (Iqbal *et al*., 2008). Natural compounds having the capacity of free radical scavenging are known for stopping lipid oxidation chain reactions (Gil *et al*., 2000).

Fruit peels have not been explored so much for their antioxidant activity in the past. There is little information on the antioxidant activity in fruit peels. The waste part of the fruit is often ignored for their potential use and this is quite true in case of the utilization of the peel (Soong and Barlow, 2004). Peels usually have higher antioxidant potential than the fruit pulp making it more important for this specific use. It is the low cost and environment friendly source of antioxidant because it will help to reduce the burden of waste material in fruits and vegetable industries. (Soong and Barlow, 2004). Fats and oil is the most important component of the diet because they provide essential fatty acids to our bodies. These fatty acids are important constituents of the many hormones in our body and regulate life essential physiological processes such as regulation of blood cholesterol, blood pressure and reproductive system (Walisiewicz-Niekbalska *et al*., 1997).

Polyunsaturated fatty acids are more prone to autoxidation which results in deteriorative changes in the lipid profile of the fats andd oils. Because of these changes the actual function can't be performed which resulted in numerous problems. Similarly, fats and oils with lipid oxidized product have characteristic off odor making it unacceptable for the consumer resulted in economic loss (Che Man and Tan, 1999). To enhance the quality of the fats and oils

antioxidants are used. Synthetic antioxidants are well established for their use such as butylated hydroxylanisole (BHA), butylated hydroxyltoluene and propyle glycate (PG). However, safety concerns from the consumers urged to discover natural source of antioxidants. These antioxidants also have carry through property resulting in a product with better health profile. Therefore, naturally occurring antioxidants have become a major area of scientific research. The present study addresses on the utilization of pomegranate peel extracts to enhance the stability of sunflower oil.

MATERIALS AND METHODS

Procurement and preparation of Raw materials

Pomegranate peel was procured from local market and sun dried. The dried peel was ground to a fine powder to pass through a 100 mesh sieve using a small laboratory grinder. Sunflower oil was procured from Kashmir oil mills, Faisalabad.

Preparation of Peel Extract

The pomegranate peel powder (5 g) was extracted for 24 hour with100 mL each of ethanol, methanol and water, respectively, on mechanical shaker (250 rpm, 37 °C, 24 hr). Then again extract the samples with respective solvents and the supernatant from each flask was filtered with Whatman filter paper No.1. The solvent from the supernatant was separated 40 °C in a rotary vacuum evaporator (EYELA, N-N series, Japan) leaving behind crude extract. The extract of each sample was weighed to determine the yield of soluble constituents and stored at 4 °C until use as described by Iqbal *et al*. (2008).

Analysis of Peel Extract

The pomegranate peel extract was analyzed for antioxidant potency through total phenolic contents, antioxidant activity by beta-carotene and free radical scavenging activity (DPPH assay) as discussed below.

Total phenolic contents

The total phenolic compounds in both extracts were determined by the Folin-Ciocalteu method (Sun *et al*., 2006). For the calibration curve 1 mL aliquots of 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 mg/mL Catechol solutions in ethanol were mixed with 5 ml of Folin-Ciocalteu reagent (diluted ten-fold) and 4 mL of sodium carbonate solution (20g/ 100mL). The absorbance was read after 30 mintues at 20 °C at 765 nm and the calibration curve was plotted using the absorbance against the respective standards. One mL of methanolic extract (10 g/100mL) was mixed with the same reagents as described above after 1 hour the absorbance was measured for the determination of total phenolics. Total content of phenolic compounds in methanol extracts in gallic acid equivalents (GAE) was calculated by the following formula:

$$C = c \times V / m$$

Where:

C = total content of phenolic compounds in mg/g plant extract, in GAE

c = the concentration of Catechol calculated from the calibration curve in mg/mL

V = the volume of extract in mL

m = the weight of plant methanolic extract in g

Determination of antioxidant activity

Determination of the antioxidant activity using a β-carotene/linoleic acid system was carried out according to the method described by Conforti *et al*. (2006). In brief, 40 mg of linoleic acid and 400 mg of Tween 20 were transferred into a flask, and 1 mL solution of β-carotene (3.34 mg/mL) in chloroform was added. Chloroform was removed by rotary evaporation at 40 °C. Then 100 mL of distilled water was added slowly to the residue and the solution was vigorously agitated to form a stable emulsion. To an aliquot of 5 mL of this emulsion, 0.2 mL of an antioxidant solution was added, and the absorbance was measured at 470 nm, immediately, against a blank consisting of the emulsion without beta-carotene. The tubes were placed in a water bath at 40 °C and the absorbance was measured every 15 min up to 60 min. The degradation rate of the extracts was calculated according to first order kinetics using following equation (Al-Saikhan *et al*., 1995)

$$\ln (a/b) \times 1/t = \text{sample degradation rate}$$

Where:

ln = the natural log

a = the initial absorbance (470 nm) at zero min

b = the absorbance (470 nm) after 60 min

t = the time (min).

The antioxidant activity (AA) was expressed as % inhibition relative to the control using following equation.

$$AA = \frac{\text{Degradation rate of control}}{\text{Degradation rate of sample}} \times 100$$

Free radical scavenging activity (DPPH assay)

DPPH radical scavenging activity of both extracts was evaluated according to the method of Conforti *et al*. (2006) with slight modification, as described below. Extract solutions were prepared by dissolving 0.025 g of dry extract in 10 mL of ethanol. A fresh solution of

DPPH in ethanol (6×10^{-5} M) was prepared before measurements. 3 mL of this solution was mixed with 77 µl (38 or 19 µl in additional assays) extract solution in 1 cm path length disposable microcuvettes (final mass ratio of extracts to DPPH was approximately 3:1, 1.5:1, 0.75:1). The samples were kept in the dark for 15 mintues at room temperature and then the decrease in absorbance was measured at 515 nm on a UV/visible light spectrophotometer (CESIL CE7200). Absorbance of blank sample containing the same amount of ethanol and DPPH solution was also measured in the same fashion. The experiment was carried out in triplicate. Radical scavenging activity was calculated as.

Reduction of absorbance (%) = [(AB - AA) / AB] × 100

Where:

AB = absorbance of blank sample (t = 0 min)

AA = absorbance of tested extract solution (t = 15 min)

Preparation of Oil and Peel Extract Blends

Sunflower oil blends (prepared by using 500, 750, 1000 ppm pomegranate peel extracts i.e. ethanol and methanol) was prepared along with commercial control by adding 200 ppm of BHT.

Analysis of blended oils

The refined sunflower oil blended with pomegranate peel extracts (ethanol and methanol) was analyzed for specific gravity, free fatty acids, peroxide value, saponification value and iodine value according to their respective procedures as described in AOCS (2003).

Statistical Analysis

The data generated during the experiments were analyzed by statistically available software (Statistic 8.1 USA) according to procedure defined by Steel *et al*. (1997).

RESULTS AND DISCUSSIONS

Extraction yield of pomegranate peel extract

The antioxidant extracts were prepared from the raw material by using ethanol, methanol and water as solvents. A significant difference between the extracts yield is observed. However, the highest extraction yield (29.48%) was recorded in methanol solvent followed by ethanol (20.80%) and water (13.37%). According to Table 2 extraction yields of three treatments showed significant differences among yield. The highest amount of extracts was obtained with methanol (29.48±0.42%) as compared with ethanol (20.80±0.33%), and water (13.37±0.38%). Wet extraction is a process in which material to be extracted is in direct contact with the solvent (Houghton and Raman, 1998). The principal behind this process is the absorption of materials having same polarity. Ethanol, methanol and acetone are the organic solvents used for this process. The results signified that the yield of extract increases with increased polarity of solvent. The result of present research is harmonized with the early findings of Wang *et al*. (1996) they reported ethanol as an effective solvent for extraction of antioxidants several other scientists carried out work on extraction of antioxidant from natural sources with varying solvents and their conditions. Brigita *et al*, (2005) found double anthocyanins and polyphenols in the extract of black current by using ethanol and methanol. In another study, Rehman (2006) found maximum extraction rate (19.87%) with methanol, acetone (15.00%) and diethyl ether (12.75%) from the different plant material.

Total phenolics determination of extracts

Polyphenols holds important consideration in health promoting properties of fruits and vegetables and estimation of its total contents has significance worth for their antioxidant potential. The total phenolic compounds from both the results were expressed as catechol equivalent/g of plant extract (Folin and Ciocalteu, 1927). The total polyphenol contents of the pomegranate peel shown in table 4.4 values indicating that the methanolic extracts having the highest total phenolic contents(TPC) contents as 3.56±0.12 mg / g as compared with ethanol 3.06±0.08 and water as 2.88±0.05 mg/g of TPC depicted in the Table 2. The statistical results for total phenolic content of pomegranate peel extracts have been presented in Table 4.3. The results showed significant differences in the total phenolic content of different extracts prepared by different solvents for the extraction of natural phytochemicals (Awika *et al*., 2003)

DPPH scavenging activity

The results of DPPH free radical scavenging activity of pomegranate peel extracts have been showed in Table 2. The results exhibit the statistically significant differences among the different treatments like methanolic, ehtanolic, water extract and BHT. The DPPH free radical scavenging activity of the pomegranate peel in the Table 2 values depicting the methanolic extracts having the highest radical scavenging activity as 82.52±0.46% as compared with ethanol 82.52±0.46 %, water 80.150±0.26 and BHT 86.467±0.73% Table 4.6. It is revealed from the means table that methanolic extract has comparable DPPH free radical scavenging activity to the reference/ standard i.e.BHT. DPPH free radical scavenging activity is one of those indicators that are important in determining antioxidant potential of selected bioactive molecules or extracts (Wu *et al*., 2004; Huang *et al*., 2005). The results

Table 1. Treatments plan

Treatments	Refined sunflower oil with different levels of pomegranate extracts
T_1	Control
T_2	500 ppm pomegranate peel ethanoic extract
T_3	750 ppm pomegranate peel ethanoic extract
T_4	1000 ppm pomegranate peel ethanoic extract
T_5	500 ppm pomegranate peel methanolic extract
T_6	750 ppm methanolic extract of pomegranate peel
T_7	1000 ppm methanolic extract of pomegranate peel
T_8	200 ppm BHT

Table 2. Means for extraction yield, total phenolics, DPPH and β-carotene assay of pomegranate peel extracts

Solvents	Extraction yield (g/kg)	Total phenolics (mg)	DPPH	β-carotene linoleate
Ethanol	20.80 ± 0.33^{b}	3.06 ± 0.08^{b}	82.52 ± 0.46^{c}	87.56 ± 0.44^{b}
Methanol	29.48 ± 0.42^{a}	3.56 ± 0.12^{a}	83.16 ± 0.72^{b}	92.21 ± 0.45^{a}
Water	13.37 ± 0.38^{c}	2.88 ± 0.05^{c}	80.15 ± 0.26^{d}	41.15 ± 0.52^{c}

Means not sharing same letter in a column differ significantly from each other ($p\leq0.01$)

Table 3. Effect of Treatments on the quality of the oil blends

Treatments	Specific gravity	Free Fatty acids (%)	Iodine Value (%)	Saponification value (mgKOH/g)	Peroxide value (meq/kg)	TBARS
T_1	0.9067 ± 0.0026^{d}	0.4667 ± 0.10^{a}	$112.99\pm3.81a$	194.58 ± 0.88^{a}	17.648 ± 1.04^{a}	0.1819 ± 0.05^{a}
T_2	0.9040 ± 0.0023^{e}	0.3208 ± 0.11^{b}	107.81 ± 3.44^{c}	193.25 ± 0.71^{c}	2.987 ± 0.74^{c}	0.1771 ± 0.04^{a}
T_3	0.9157 ± 0.0002^{b}	0.3024 ± 0.11^{c}	103.77 ± 3.70^{e}	193.67 ± 0.62^{bc}	3.116 ± 0.78^{b}	0.1461 ± 0.03^{bc}
T_4	0.9186 ± 0.0008^{a}	0.2752 ± 0.11^{d}	98.06 ± 3.73^{f}	193.17 ± 0.65^{cd}	2.543 ± 0.51^{e}	0.1322 ± 0.03^{d}
T_5	0.9090 ± 0.0018^{c}	0.3258 ± 0.11^{b}	109.62 ± 3.00^{b}	194.00 ± 0.68^{ab}	2.944 ± 0.71^{c}	0.1789 ± 0.04^{a}
T_6	0.9157 ± 0.0008^{b}	0.3008 ± 0.10^{c}	105.55 ± 3.20^{d}	194.08 ± 0.66^{ab}	2.683 ± 0.63^{d}	0.1556 ± 0.03^{b}
T_7	0.9187 ± 0.0010^{a}	0.2599 ± 0.11^{e}	108.05 ± 3.51^{c}	192.50 ± 0.56^{d}	2.067 ± 0.44^{g}	0.1203 ± 0.02^{e}
T_8	0.9145 ± 0.0002^{b}	0.2925 ± 0.11^{c}	105.40 ± 3.58^{d}	194.58 ± 0.64^{a}	2.367 ± 0.48^{f}	0.1452 ± 0.03^{c}

Means not sharing same letter in a column differ significantly from each other ($p\leq0.01$)

Table 4. Effect of Storage on the quality of the oil blends

Parameters	Storage days			
	0	10	20	30
Specific gravity	0.9103 ± 0.0034^{c}	0.9132 ± 0.0017^{b}	0.9126 ± 0.0014^{b}	0.9153 ± 0.0003^{a}
Free Fatty acids (%)	0.0813 ± 0.03^{d}	0.2134 ± 0.02^{c}	0.4204 ± 0.02^{b}	0.5570 ± 0.03^{a}
Iodine Value (%)	121.38 ± 1.04^{a}	109.20 ± 1.75^{b}	101.97 ± 1.46^{c}	93.08 ± 1.22^{d}
Saponification value (mgKOH/g)	190.96 ± 0.18^{d}	192.88 ± 0.19^{c}	194.75 ± 0.22^{b}	196.33 ± 0.32^{a}
Peroxide value (meq/kg)	1.9717 ± 1.33^{d}	3.4517 ± 1.50^{c}	5.6154 ± 1.61^{b}	7.1388 ± 1.72^{a}
TBARS	0.0015 ± 0.00^{d}	0.1288 ± 0.01^{c}	0.2312 ± 0.02^{b}	0.2571 ± 0.01^{a}

Means not sharing same letter differ in a row significantly from each other ($p\leq0.01$)

of current study were in agreement of the previous findings, and show that this method is simple and effective for antioxidant capacity evaluation, as found by other existing assays carried out for the same purpose. Likewise, another study conducted by Zhang *et al*. (2010) The IC_{50} values in the DPPH assay obtained for rosemary extracts 25, rosemary extracts 60, rosemary extracts 98, BHA, BHT and TBHQ were 0.31±0.003, 0.22±0.004, 0.21±0.001, 0.20±0.006, 0.42±0.010, and 0.09±0.002 mg/ml, respectively. From the above results it revealed that the similar pattern was found in present study. The results were varied due to different laboratory scale reagent were used.

Antioxidant activity using β-carotene linoleate model system

Antioxidant activity of pomegranate peel extract of different solvents have been presented in Table (2) showed significant differences in the Antioxidant activity of pomegranate peel extract. The results depicted that the antioxidant activity via beta carotene bleaching assay ranged from 92.21±0.45 to 41.15±0.52 %. The highest activity was found in methanolic extract (92.21±0.45) followed by ethannolic extract (87.56±0.44) and water (41.15±0.52). Matthaus *et al*., (2002) found that the antioxidant activity determined through beta carotene bleaching method of the sunflower was almost 70% whereas another study was conduct on the seeds revealed antioxidant level up to 72.9% (Velioglu *et al*., 1998). The results are compareable with the earlier studies conducted by (Taga *et al*., 1984; Zhang and Hamauzu 2004). In other study of Kähkönen *et al*. (1999) used similar the carrot was found to have 52% on dry weight base. The different results are probably not only due to the use of different extraction methods and cultivars used but extracts also used in the measurements of antioxidant activity. Zhang *et al*. (2010) were also concluded that the rosemary extract having high content of carnosic acid (phenolic compound) attributed toward stability of sunflower oil. But the activity levels were varied among different synthetic and natural compounds were tested in sunflower oil, these were described as L-ascorbic acid > rosemary extract 98 > TBHQ > BHA > rosemary extract 60 > BHT > rosemary extract 25.

Storage stability of sunflower oil

Free fatty acids

Data thus obtained was analyzed statistically and analysis of variance revealed that treatments and storage periods and their interactions were highly significantly altered free fatty acid contents. Table 3 indicated that treatments were significantly different to each other and on overall basis T_1 (Control) gave maximum FFA contents (0.4667% oleic acid) and rest of all others treatments tend to reduce the free fatty acid contents and FFA contents were recorded in the range of 0.2599 to 0.3258%. BHT produced FFA contents (0.2925±0.11) that were statistically different from T_1 followed by T_2, T_3, T_4 and T_5 with mean FFA contents. Among all treatments containing antioxidant rich extracts only T_7 was more effective as compared to BHT and they less tend to produce FFA contents of. Among the all treatments T_7 proved best and the concentration of pomegranate peel at that treatment proved effective. It is depicted that oil samples containing T_7 gave maximum protection against FFA production followed by BHT and T_3, T_4 and T_5 and T_6 behaved statistically similar and they inhibited FFA production while among treated groups T_1 and T_2 provided least protection. The results of present study are in line with the findings of Iqbal and Bhanger, (2007) for FFA content whcih showed increasing trend with increased storage time. Highest free fatty acids were observed in the control treatment. However, increase in FFA was observed after 7 to 8 days of storage.

Iodine value

The means (Table 3) indicated that treatments were significantly different to each other and on overall basis T_1 (Control) gave maximum iodine value (112.99) and rest of all others treatments tend to reduce the iodine value, which was recorded in the range of 98.6 to 112.99. BHT produced iodine value was observed (105.40) that was statistically similar to T_2. Among treatments containing antioxidant rich extracts only T_4 was more effective as compared to BHT and followed the T_3, T_8, T_7, T_2, T_1. It is depicted that oil samples containing T_4 gave minimum iodine value which means it is more stable than rest of treatments. Study conducted by Iqbal and Bhanger, (2007) found that the iodine value increased with increase in storage time. Current study, harmonized with the finding of Rehman *et al*. (2006). Potato peel extract was evaluated as a antioxidant in refined soy oil at 25 and 45ºC. Iodine values of soy bean oil containing 1600 and 2400 ppm of potato peel extract were 71 and 77, respectively, were obtained which were higher than the control samples of oil. However, iodine values for soy bean oil treated with 200 ppm of BHA and BHT were 80 and 84, respectively.

Specific gravity

The results for the specific gravity showed significant effect on specific gravity of the sunflower oil (Table 3), exhibited that T_4 showed maximum specific gravity (0.9186) and rest of all others treatments tend to increase the specific gravity, which was recorded in the range of 0.9067 to 0.9186. BHT containing sunflower oil revealed specific gravity (0.9145) that was statistically similar to T_3 and T_6. Among all treatments containing antioxidant rich extracts only T_3 and T_6 were more effective as compared to BHT. It is evident from the results that specific gravity of sunflower oil increased with storage time with and without the pomegranate peel extract addition to it. This increase in values may reflect the incident of polymerization, which makes the oil denser. These results are harmonized with the findings of Anjum *et al*. (2006) and Amoo (2004) who reported that the increase in specific gravity in oil from roasted sunflower and coconut seeds.

Saponification value

The data showing the saponification value of sunflower oil are presented in Table 3. From statistical analysis, results revealed significant increase in saponification values. The saponification values were measured by titration method and comparison of means for the saponification values of sunflower oil is given in Table 4.18 which showed a minimum value of 192.5 for T_7 sample and maximum value of 194.58 for the treatment T_1 (110 ºC, 4% moisture) treatment. The data indicated an increasing trend of saponification values with increase in storage time, whereas, the saponification value was significantly attributed to reduce by application of the pomegranate peel extract in sunflower oil. Pomegranate peel extract may inhibit the increase of the saponification value due to high antioxidant phytochemicals present in it. It is exhibited from the results that saponification value of sunflower oil from temperature and moisture conditioned seeds increased with conditioning temperatures while moisture has inverse effective in this regard. These results are relevant to the findings of Anjum *et al*. (2006) and Soetaredjo *et al*. (2008) who showed increase in sponification values of sunflower oil and neem oil with increase in conditioning temperatures. Amoo (2004) also observed an increase in sponification value in coconut oil when coconut seeds were roasted before oil extraction.

Peroxide value

Peroxide value (POV) is indicator of oil rancidity and as oxidation continues it tends to produced free radicals and interacting with other molecules to produce aldehydes and ketonic bodies that yields odd flavor. Peroxide value was significantly affected with the treatments and storage periods. Interactive effect of both factors affected the peroxide value significantly. Table 3 indicated that treatments with antioxidants addition gave less POV as compared to control. Pooled means for treatments revealed that T_1 (Control) gave maximum POV (17.648%leic acid). T_7 and T_8 gave lowest POV value of 2.067 and 2.367% respectively. Oil samples treated with BHT also inhibited lipid per-oxidation with pooled POV value of 2.367%. Time scale measurement of POV revealed that with the passage of time POV values tends to increase and maximum POV value was recorded at the end of the study while lowest was recorded at 0 day of storage. Iqbal and Bhanger, (2007) recorded peroxide value in the range of 60.71 to 98.39 meq/kg with maximum 170 meq/ kg. In a similar study, garlic extract, controlled peroxide value appreciably; revealing good antioxidant efficacy in stabilization of oil (Shahidi *et al*., 1995).

Thiobarbituric acid reactive substances (TBARS) Assay

TBA value is indicator of oxidative deterioration. It is significantly affected with the treatments and storage periods. Interactive effect of both factors affected the peroxide value significantly Table 3. Analysis of variance for the TBA value indicated significant difference among the treatments between the storage periods and interaction between them was also proved significant. Data showing means is presented in Table 3 that indicated that treatments with antioxidants addition give rise to reduce TBA value as compared to control. Pooled means for treatments revealed that T_1 (Control) gave maximum TBA value (0.1819). Oil samples containing BHT gave produced amounts of TBA products (0.1452). Treatments containing extracts from pomegranate peel extract results in lower amounts of TBA products. Among pomegranate peel extract treatments T_7 give maximum best results (0.1203) followed by the T_4 (0.1322) TBA value. Storage has profound effect on TBA value and it increased with increase in storage and least TBA value was observed and while maximum TBA value was obtained at 90 days of storage. Interactive effects revealed that in control TBA value changes from 2.8 to 4.57 while in oil samples with BHT ranged from 0.56 to 1.17. Another study was done to utilize

pistachio hull extract along with commercial antioxidants to determine their relative effectiveness for enhancing the quality of soy bean oil (Goli *et al*, (2005). The results showed that all samples with peel extract were more stable (0.02-0.06%) than the controls. Howere, it was also noted that synergistic effect was more effective than the single use of antioxidants.

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Quality evaluation of ice cream prepared with *phoenix dactylifera* syrup as a substitute of sugar

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ABSTRACT

Ice cream samples were prepared with different concentration of date syrup (0, 25, 50, 75 and 100%). The ice cream sample having Cremodan was kept as reference standard. Ice cream was analyzed for textural and compositional analysis for fresh and physico-chemical and sensory characteristics at 0, 15, 30, 45 and 60 days storage. The increase in date syrup percent resulted in increase of viscosity and over run values while melt down value was decreased. During 60 days of storage, pH, over run, and melt down decreased while viscosity, standup time and acidity increased. On sensory evaluation, highest score was awarded to ice cream sample prepared with 50% date syrup. Progressive deterioration was found in all sensory characteristics but during storage non-significant effect was observed on flavor, texture, sweetness and overall acceptability. It was found that ice cream with better quality can be made using date syrup with good sweetness level.

Key words: Date, Syrup, Sugar, Replacer, Sweetness

INTRODUCTION

Ice cream is a nutritious, palatable and comparatively less expensive food. No other food attain much popularity and has a fascinating appeal and form as ice cream (Goff, 2002). Ice cream is a very nutritious product (Goff and Hartel, 2013). In ice cream formulation different varieties of sweeteners have been used. In ice cream production sucrose is the most frequently used sweetener due to economic and rheological reasons. Sucrose has many drawbacks due to its high glycemic index (GI) which is correlated with diabetes mellitus, metabolic syndrome, hypertension, obesity and dental caries (Trasoff *et al*., 2008). That's why in these days, artificial sweeteners have been suggested to be substitutes for sucrose. Artificial sweeteners produce minute or no calories but different safety concern has been raised, such as teratogenicity, carcinogenicity and also involvement with some vascular diseases.

The *Phoenix dactylifera* (date palm) is one of humankind's oldest cultivated plants. As food date fruit has been used for 6000 years. Due to its excellent nutritional, health, and economic value with the addition of its environmental and aesthetic benefits it could be used for generations (El-Nagga and Abd El-Tawab, 2012). Date fruit provide beneficial anticipation for aggressive hunger and diseases. Due to its rich composition of sugars, carbohydrates, dietary fiber, fatty acids, vitamins, protein and amino acids, it become more important in human nutrition (Alanazi, 2010). Worldwide fresh dates having more than two thousand different varieties (Razavi *et al*. 2007). It is very palatable, highly nutritious and provides carbohydrates, vitamins and minerals (Sindhu *et al*., 2003). Dates are a source of fibers, calcium, magnesium and phosphorus (Ali Mohammed & Khamis, 2004). Fresh date provide 125 kilo calories of energy in each 100 grams which is equivalent to one fourth gram of sugar (Cardoso and Bolini, 2007).

These days due to healthy life style, consumption of healthy diets with low sucrose and low glycemic index trend is increase. Therefore, focus of many researchers in food technology to introducing new food items with little or no calories. Worldwide the production of ice cream increased as well as consumption, as highly nutritive frozen dessert, parallels with improved trend in prevalence of diabetes mellitus, obesity and dental caries (Mahan and Sylvia, 2008; Jenkins *et al*., 2008). Froze desserts especially ice cream widely liked by children and some other age groups and also included in food group of many families in worldwide. Ice cream production with low glycemic index will be beneficial to control the happening of life style related diseases. The objective of this study was to inspect glycemic index, physicochemical and sensory characteristics of ice cream prepared with different concentration of sucrose and date syrup and also introduce a novel date syrup based ice cream.

MATERIAL AND METHODS

Procurement of raw materials

The milk, milk cream fresh date and other ingredients such as sugar, skim milk powder, stabilizer (cremodan) artificial flavor and food grade color was purchased from the local market. The following combination of date syrup and sugar were used accordingly in Table 1.

Preparation of date syrup

Fresh dates was purchased from local market, washed, de-stoned, cut into pieces and boiled with water for 10 minutes. After that resultant slurry was blended for 5 minutes and filtered through muslin cloth with a hand press. The residue pulp was rewashed with sufficient amount of hot water and filtered again. The collected raw date juice was centrifuged for 5 minutes at 10,000 rpm. The clear extract was concentrated under vacuum using rotary evaporator at 75^0C to obtain one third of the total extracted volume. After it, syrup was cooked up to 70^0 brix and filled in pre-sterilized glass bottles and stored at ambient temperature for using its different percentages in formulation of ice cream.

Preparation of ice cream mix

The ingredients like stabilizer, date syrup, sugar and skimmed milk powder were weighed and mixed with liquid milk and milk cream by constant mechanical stirring. The mixture was pasteurized for 30 min at 72^0C and then homogenization was done with electric homogenizer (U/MIN 7000 Type B-1 Elek tromischer Made in Germany). After the homogenization, ageing was perfumed for 5 hours at 4 ^{0}C. The mixture was further subjected along with whipping of air in ice cream at low temperature of –1 to -9^0C (Schmidt, 2004). after that ice cream was filled in 100 or 150 mL disposable cups and further proceed toward hardening unit at -28 to -30^0C for 24h for physico-chemical and sensory evaluation. After the preparation ice cream was stored in freezer for 60 days at –24 °C.

Compositional analysis of ice cream

Moisture of ice cream samples was estimated by AOAC (2003). Lactose was evaluated by method as given in AOAC (2000). Fat content of ice cream samples were determine by Gerber method AOAC (2003). The total nitrogen was determined by the kjeldhal's method as given by IDF (2006). Ash content in ice cream was estimated by charring of the sample by the method of AOAC (2003). Lane and Eynon method was used to estimate the reducing sugars as described in method No. 925.36 of AOAC (2000).

Physico-chemical and textural analysis

Ice cream samples were examined for physico-chemical and textural analysis at 0, 15, 30, 45, and 60 days of storage period. The pH of ice cream was determine by using the electronic digital pH meter (Inolab WTW series 720). Acidity of prepared ice cream was measured by following titration method mention in AOAC (2003). Viscosity measurement of ice cream samples was done by following the way described by (Farrag *et al.*, 2010). Standup time of prepared ice cream samples was estimated according to method narrated by Bhandari (2001). Over run of the product was estimated as per AOAC (2003). In case of ice cream meltdown test is very important because it provides us knowledge about the firmness of ice cream at the time of consumption. Melt down of the product was estimated by the method described by (Santana *et al*., 2011).

Sensory Evaluation

Sensory evaluation based on ice crystals, flavor, texture, sweetness, hardness, appearance and overall acceptability was conducted using 9-point hedonic scale according to the method described by (Meilgaard *et al*. 2007).

Measurement of Glycemic Index

In the study ten participants were recruited for glycemic index measurement. Selection were done on the base of following criteria: health, post-prandial glucose levels and fasting was normal according to physical examination and not receiving any medicine or supplements during past three months. The participants were advised to maintain overnight fasting for ten hours. For reference glycemic response calculation, the participants were then asked to take 50 kg pure glucose and plasma glucose was tested. Capillary blood glucose analyzer was used to determined blood glucose levels. The experiments were repeated for two consecutive days using soft ice cream and date syrup ice cream. Finally, Glycemic index was calculated by using standard equation.

Statistical Analysis

For each parameter analysis of variance was done to find out the level of significance according to the method as illustrate by (Steel *et al*. 1997).

RESULTS AND DISCUSSION

Compositional analysis of ice cream

The compositional analysis of ice cream was done for fresh ice cream. The results expose that significant

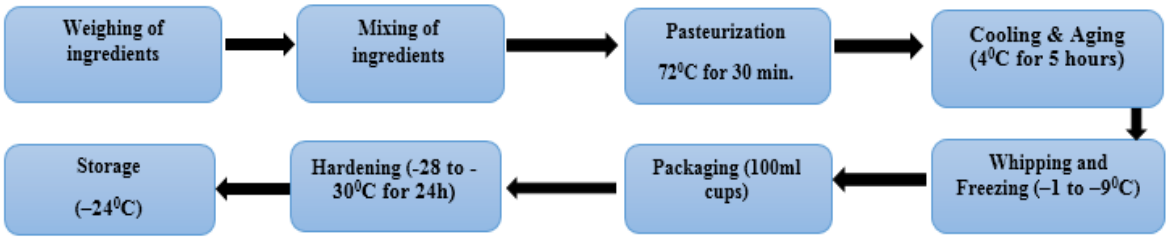

Fig. 1. Flow diagram of the preparation of date syrup based ice cream

Table 1. Different concentration of date syrup for ice cream preparation

Treatment	Sugar (%)	Date syrup (%)	Total (%)
T_0	100	0	100
T_1	75	25	100
T_2	50	50	100
T_3	25	75	100
T_4	0	100	100

Table 2. Compositional Analysis of fresh ice cream prepared by different concentration of date syrup

Characteristics/Treatments	T_0	T_1	T_2	T_3	T_4
Moisture (%)	58.78±0.15e	61.36±0.32d	65.17±0.10c	67.31±0.92b	69.20±0.08a
Protein (%)	3.33±0.02e	3.93±0.05d	4.14±0.03c	4.40±0.2b	4.67±0.42a
Fat content (%)	10.09±0.01bc	10.10±0.02a	10.09±0.04bc	10.10±0.01ab	10.10±0.01ab
Ash content (%)	0.61±0.01e	0.78±0.02d	0.83±0.025c	0.94±0.01b	1.01±0.025a
Lactose content (%)	5.51±0.10e	5.53±0.09d	5.55±0.14c	5.57±0.08b	5.6±0.63a
Reducing sugar (%)	3.94±0.10e	5.43±0.09d	7.12±0.12c	8.64±0.08b	10.18±0.63a
Non-reducing sugar (%)	13.13±0.10a	11.18±0.09b	9.25±0.14c	7.30±0.08d	5.40±0.63e

Table 3. Comparison of means for physico-chemical and textural analysis influenced by various treatment

Characteristics/Treatments	T_0	T_1	T_2	T_3	T_4
Overrun (%)	50.70±0.35	51.96±0.0.62	53.30±0.63	54.74±0.65	55.90±0.73
Meltdown time (mL/10 min.)	28.86±0.10	24.94±0.12	22.14±0.11	19.96±0.15	17.56±0.13
Viscosity (cP)	2254.5±18	2260.9±21	2290.9±23	2340.6±15	2403.5±18
Standup time (min.)	11.42±0.05	11.68±0.10	12.40±0.08	12.76±0.12	12.96±0.14
pH (%)	6.71±0.05	6.64±0.07	6.55±0.10	6.51±0.12	6.43±0.10
Acidity (%)	0.207±0.005	0.214±0.001	0.219±0.002	0.223±0.003	0.228±0.003

Table 4. Comparison of means for physic-chemical and textural analysis as influenced by storage period

Characteristics/storage	0	15	30	45	60
Overrun (%)	54.32±0.45	53.76±0.53	52.36±0.64	51.80±0.64	50.80±0.69
Meltdown (mL/10 min)	25.84±0.12	24.44±0.16	22.54±0.17	21.14±0.14	19.32±0.20
Viscosity (cP)	2105.9±25	2201.9±28	2290.8±32	2394.1±18	2560.1±20
Standup time (min.)	10.02±0.05	10.90±0.08	11.92±0.11	13.02±0.14	14.02±0.15
pH (%)	6.72±0.05	6.66±0.08	6.59±0.10	6.50±0.11	6.39±0.13
Acidity (%)	0.208±0.003	0.215±0.002	0.218±0.001	0.222±0.004	0.227±0.003

Table 5. Comparison of means for sensory evaluation as influenced by treatment

Characteristics/Treatments	T_0	T_1	T_2	T_3	T_4
Flavor	7.97±0.81	8.14±0.62	8.33±0.71	8.02±0.65	7.81±0.59
Texture	7.90±0.71	7.72±0.56	8.04±0.78	7.66±0.65	7.34±0.78
Ice crystal	8.02±0.61	7.84b±0.72	7.95±0.65	7.43±0.69	7.10±0.65
Sweetness	7.76±0.74	8.07±0.81	8.17±0.58	7.35±0.56	7.08±0.67
Appearance	6.93±0.64	7.04±0.59	7.56±0.75	7.25±0.61	6.92±0.59
Hardness	7.74±0.76	7.60±0.68	7.85±0.73	7.44±0.70	7.28v±0.60
Over all acceptability	7.75±0.57	8.05±0.76	8.26±0.84	7.97±0.75	7.84±0.63

Table 6. Comparison of means for sensory evaluation as influenced by storage period

Characteristics/storage days	0	15	30	45	60
Flavor	8.23±0.65	8.25±0.75	8.06±0.55	7.92±0.59	7.95±0.63
Texture	7.92±0.60	7.83±0.81	7.76±0.58	7.68±0.65	7.57±0.71
Ice crystal	8.14±0.71	7.92±0.56	7.74±0.59	7.43±0.63	7.38±0.64
Sweetness	7.78±0.75	7.66±0.60	7.58±0.50	7.52±0.57	7.59±0.62
Appearance	7.63±0.83	7.33±067	7.11±0.71	6.84±0.74	6.67±0.78
Hardness	7.85±0.67	7.75±0.69	7.63±0.71	7.45±0.73	7.34±0.65
Over all acceptability	8.23±0.65	8.04±0.71	7.96±0.68	7.83±0.70	7.62±0.84

effect on moisture, protein, ash content, reducing sugar and non-reducing sugar by ice cream samples having different combination of date syrup and sugar except fat content and lactose content having non-significant effects on treatments (Table 2). Data revealed that moisture contents of all the treatments vary significantly ($P<0.05$) and ranged between 58.78-69.20% among different treatments. The highest mean value was observed in T_4 (69.20%) while lowest was observed in T_0. The similar findings for approximate analysis of moisture level 62.84 to 63.90 % was reported in ice cream with two jambolana fruit levels and 65% moisture in plain ice cream (Murtaza *et al*., 2004). Protein contents of date syrup based ice cream ranged from 3.33- 4.67. Statistical analysis showed that it varied significantly ($P< 0.05$) in all the treatments. The highest mean value for protein content was observed in T_4 (4.67%) that might be due to protein present in date syrup (2.5%) and the lowest value was observed in T_0 (3.33%). Murtaza *et al*. (2004) reported that the addition of fig have significant ($P< 0.05$) effect on the protein content of ice cream.

It is obvious from statistical results fat content differed non-significantly ($P>0.05$) among different treatments. There was a similar fat results (10.10%) for T_1, T_3, T_4 while for T_0, T_2 (10.09%) was observed. Addition of fig fruit have non-significant ($P>0.05$) effect on the fat content of ice cream reported by Murtaza *et al*. (2004). The ash is an inorganic residue obtained after the removal of water and organic matter by heating in the presence of oxidizing agents. It actually measures the total amount of mineral in the food. Statistical analysis showed that ash content varied significantly ($P< 0.05$) in all the treatments. The highest mean value was observed in T_4 (1.01%) and the lowest value was observed in T_0. Addition of fig fruit have significant ($P<0.05$) effect on the ash content and increase by increasing the percentage of fig fruit reported by Murtaza *et al*. (2004). Lactose contents of date syrup based ice cream ranged between 5.51-5.56 among different treatments. Results showed that lactose contents of date syrup ice cream varied non-significantly ($p> 0.05$) in all the treatments. The range

Viscosity is the resistance to flow and is commonly measured in centipoise (cP). The factors which can affect the viscosity of ice cream include temperature, type, concentration and fat globule size (Mudgil *et al*. 2011). Moreover, protein hydration can also increase the viscosity. Viscosity is a factor used to determine the date syrup aggregation, creaming, incorporation of air, freezing rate, flow condition. It can also provide mouth feel and flavor to the ice cream (Hematyar *et al*. 2012). The statistical outcomes showed that treatments highly significant ($P<0.01$) effects on viscosity of ice cream like highest mean value was observed in T_4 (2403.5cp) and lowest mean value was observed in T_1 (2254.5cp) (Table 3). While storage period showed

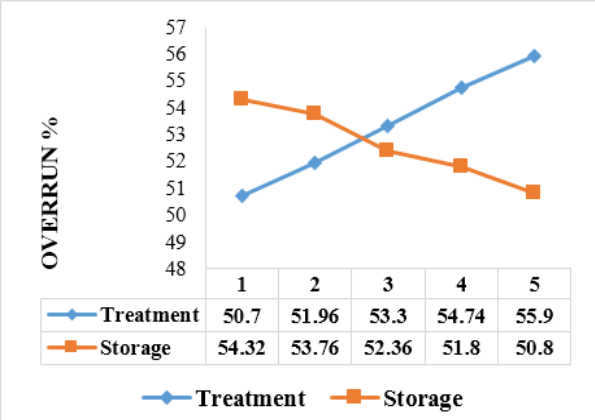

Fig. 2. Means comparison for overrun (%) of different treatment of date syrup and storage days

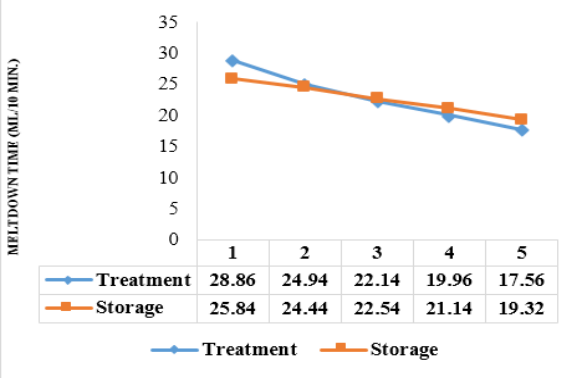

Fig. 3.Means for meltdown time (mL/10 min) for different treatment of date syrup and storage days

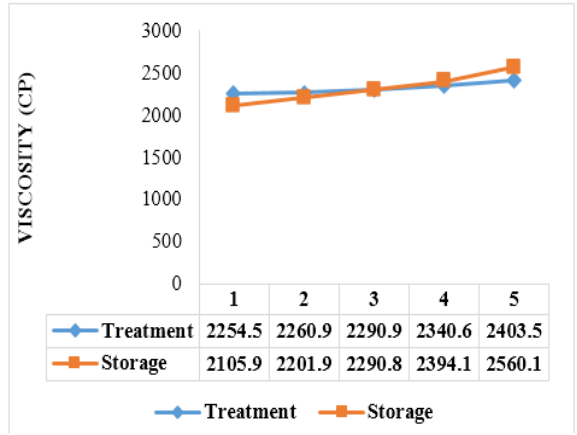

Fig. 4. Means for Viscosity (cP) for different treatments of date syrup and storage period

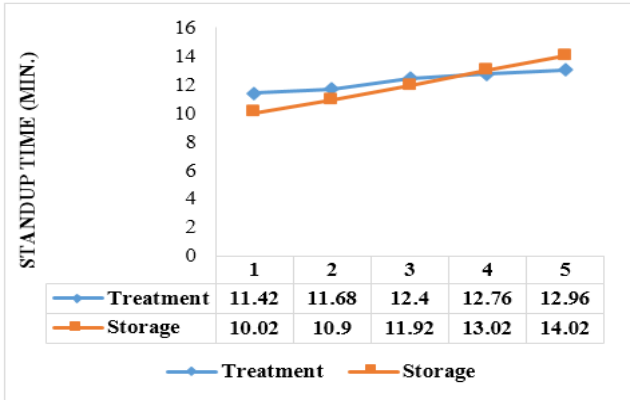

Fig. 5. Means for standup time for different treatments of date syrup and storage days

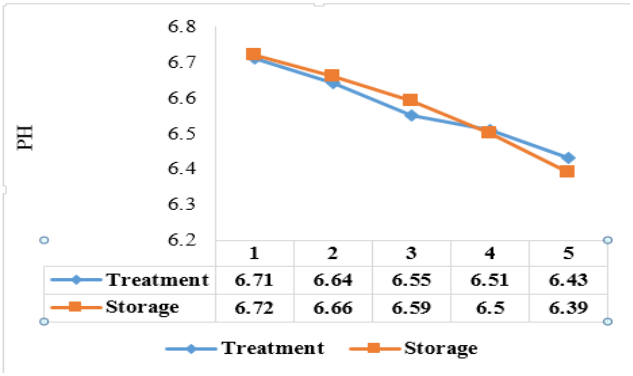

Fig. 6. Means for pH for different treatments of date syrup and storage

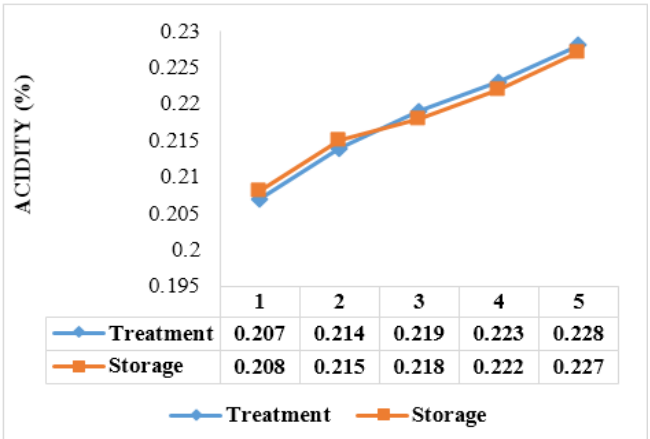

Fig. 7. Means for acidity (%) for different treatments of date syrup and storage

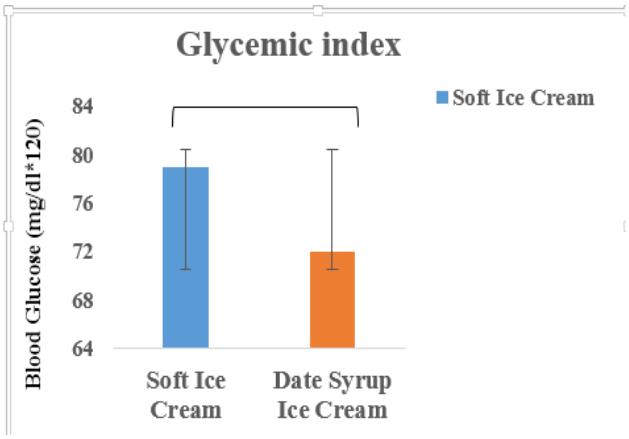

Fig. 8. (A) Blood glucose response after consumption of soft and date syrup ice cream during 120 minutes (B) glycemic index of soft and date syrup ice cream

significant and interaction showed non-significant. The viscosity was gradually increase in all samples during storage (Table 4). The outcomes are supported by the results of (Kaya and Tekin, 2001).

Standup time

Results revealed that treatments and storage period showed significant ($p<0.05$) while their interaction showed non-significant effect ($p>0.05$) on standup time of date syrup ice cream. (Table 3). The highest mean value was recorded in T_4 (12.96) and lowest standup time was observed in T_0. The period which elapsed before the first drop of melted ice cream fell was noted for each sample. Ice cream with high melting quality begins to show definite melting within 10-15 minutes when placed at room temperature. The standup time for normal ice cream is 13 minutes at 20°C (Marshall & Arbuckle, 1996). During storage period observed that significant increase happened in standup time (Table 4).

pH and acidity

Statistically, pH and acidity have significant ($P<0.005$) effect on treatments and storage due to the varying concentrations of date syrup. In dairy products the pH has direct influence on the flavor perception. It is influenced by the compositional and biochemical changes during the storage period (Kanbakan *et al*. 2004). The highest mean value 6.71% of pH was recorded in control while date syrup ice cream have comparatively low pH (Table 3). During 60 days of storage, there was a gradual increase in acidity of all the ice cream samples (Table 4).

Sensory evaluation

Ice cream samples were evaluated organoleptically on the basis of flavor, texture, ice crystals, sweetness, appearance, hardness and overall acceptability. When date syrup in the form of paste was added into the ice cream mix enhanced creamy appearance, unique taste and flavor. The highest score for flavor was awarded to ice cream sample T_2. Scores differed significantly ($p<0.005$) for all samples with varying amount of date syrup. The highest scores for sensory characteristics was awarded to ice cream sample having 50% date syrup followed by the sample having 25% date syrup (Table 5). Didrikson (1993) reported that the type of ice cream base, heat treatment of ice cream mix, pH and overrun of the product affect the color stability of the ice cream. But during the storage period of all ice cream samples, the scores for all the sensory characteristics was found gradually decrease. Significant ($P<0.005$) effects of storage on appearance, ice crystal and hardness of the ice cream samples. However, non-significant ($P>0.005$) effect on flavor, texture, sweetness and overall acceptability was found (Table 6). The formation of the ice crystals also indicates the presence of high amounts of the sugar and low level of fat. These ice crystals will form sandiness in the ice cream. The deterioration during storage with the passage of time in sensory characteristics of ice cream was reported by Palich (1994).

Effects of date syrup as a sugar replacer on glycemic index measurement

Healthy adults with average age of 22.4± 4.01 years were selected for this test. Average body mass index of the contributor was 22.5± 4.32.Ice cream manufacturing with sucrose and with date syrup, the mean glycemic index was measured as 79.06± 4.01 and 72.51±4.6. Postprandial blood glucose level is affected by both quality and quantity of carbohydrate. Data clarify a positive relation between increased dietary Glycemic Index, amount of calorie and risk for coronary heart disease (Mahan & Sylvia, 2008). Judging from the remarkable reduction in Glycemic Index of date syrup based Ice Creams and in line with previous findings (Tahvonena *et al*., 2006). We suggested that date syrup substitution with sucrose brings a new relatively healthy choice for food basket of families with high risk of life style related diseases including diabetes mellitus.

CONCLUSION

The main purpose of this research was to develop a new ice cream variety with pleasant flavor and taste.

Date syrup possess sweetness and enhance ice cream taste which makes them a perfect ingredient for ice cream manufacture. Being fat free and rich in mouth feel, it can replace sucrose and provide delicate flavor to the ice cream. Due to the more health benefits of date fruit we finalize that date syrup with sucrose is may be a satisfactory choice to produce a low calorie and glycemic index ice creams with no menacing effect on physico-chemical and sensory properties. Hence ice cream with better quality can be made using date syrup with good sweetness level.

Conflict of interest

The authors declare no conflict of interest.

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It is elucidate that all the authors do not have any affiliations with in any organization for any financial interest in the subject materials discussed in this manuscript. There is no conflict of interest among authors regarding the submission of this research work.

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