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Effect of Exo-polysaccharides from different *Penicillium* sp. on quality of cloudy apple juice during storage

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ABSTRACT

In the present study, effect of exopolysaccharides of six *Penicillium* species on the quality parameters including: cloud stability, phenolic degradation, antioxidant activity, volatile and non-volatile compounds of cloudy apple juice were evaluated during storage for two weeks at 4 ^{O}C. The added exopolysaccharides showed increase in cloud stability and exhibited a dose-dependent free radical scavenging activity as shown by their DPPH radical and β-carotene assays. The volatile and non-volatile components of the selected treatment (*Eup. pinetorum* ATCC 14770) were performed using gas chromatography as well as Gas chromatography-Mass spectrometry and High performance liquid chromatography, respectively. Twenty-two volatiles, namely 16 esters, 2 alcohols, 2 aldehydes, 1 ketone and 1 monoterpene hydrocarbons were identified in the investigated samples. A total of 10 phenolic compounds were identified, the predominant compound was chlorogenic acid. This study revealed the value of exopolysaccharides as natural clouding and flavour stabilizer.

KEYWORDS

Exo-Polysaccharides;
Penicillium sp.;
Cloudy apple juice;
Antioxidant;
Volatile compounds.

INTRODUCTION

Cloudy or unclarified apple juice has increasing market due to its superior sensory and nutritional qualities. This natural food product has lots of pulp which enhances its sensory properties and provides fiber and nutrients that may be lacking in clarified juices[13]. Current research has shown some advantages of consuming cloudy apple juice compared to clear juice[23], indicating that this product may be more beneficial to human health than clear apple juice.

Nagel[28] sdescribes a cloudy apple juice as a light, whitish yellow juice showing definite cloudiness, which shows no sedimentation, is full bodied and juicy, but has no astringent or bitter taste. The main problem with cloudy apple juice production is the assurance of colour and cloud stability, which are related to enzyme activities. The discolouring of cloudy apple juice results from enzymatic browning, which is caused by the action of polyphenol oxidase catalyzing oxidation of phenolic compounds. The control of endogenous pectin methylesterase activity is crucial for the cloud stability of cloudy juices.

Many procedures were proclaimed, including the use of ascorbic acid and nitrogen, blanching of pulp, and controlled pectolytic enzyme treatment. Another

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approach for the prevention of enzymatic browning of fruit juices has been the use of pH variation, heat treatment and antibrowning agents[39]. Although these methods may be effective in retarding or preventing browning, they also have undesirable effects such as ûavour damage, nutritional losses, chemical concern, and colour alterations.

Recently, microbial polysaccharides have attracted increasing attention because of their effective antioxidant activity. Many studies have shown that microbial polysaccharides improve the activity of antioxidant enzymes, scavenge free radicals, and inhibit lipid oxidation[35]. Polysaccharides are widely found in animals, plants, and microorganisms that have numerous bio-activities, e.g. antitumour, anticancer, antiviral, anticoagulant, and immunological activities. Fungi are an important resources of natural bioactive compounds such as alkaloids, terpenoids, steroids, quinones, isocoumarins, lignans, phenylpropanoids, phenols and polysaccharides[2] with a variety of bioactivities, and have varied applicable aspects in agriculture, medicine and food industry.

In our previous study[16] we had perform screening the available *Penicillium* species for their exo- and endo-polysaccharides producing ability and selecting the most potent species '*Eup. pinetorum'* and then determining the optimum environmental conditions for its polysaccharides production in submerged cultures. In addition, we attempted to investigate the effect of selected species exopolysaccharides on flavour compounds of cloudy apple juice.

Little is known so far about the antioxidant effects of fungal polysaccharides in real food system, especially, the antioxidant activities of the polysaccharides from *Penicillium* species which have been rarely reported[7]. Therefore, this study aimed to investigate the effect of six *Penicillium* isolates polysaccharides on cloud stability, phenolic degradation, antioxidant, volatile and non-volatile of cloudy apple juice during storage for two weeks at refrigerator.

MATERIAL AND METHODS

Materials

(a) Fungal species

The *Penicillium* species were obtained from NRRL (Agicultural Research Culture Collection) and ATCC (American Type Culture Collection).

(b) Fruits

Fresh apples (*Malus domestica* cv. Anna) were purchased from a local fruit market, Dokki, Giza, Egypt during 2013-2014 seasons.

(c) Chemicals

Folin–Ciocalteu's reagent, linoleic acid, β-carotene, DPPH (1,1'-diphenyl-2-picryhydrazyl), Butylated hydroxy toluene (BHT), *tert*-Butylated hydroxyl qunione (TBHQ), Gallic acid, 3,5-dinitrosalicylic acid (DNS), D-glucose were purchased from Sigma (Germany). All other chemicals such as absolute ethanol, sulphuric acid, barium carbonate (anhydrous), phenol crystal, chloroform, Tween 20, toluene, sodium carbonate, glucose, yeast extract, peptone, KH_2PO_4, $MgSO_4.7H_2O$ were of analytical grade.

Methods

(a) Fungal cultivation

Cultures were maintained at 4°C on potato dextrose agar (PDA) plates with periodic subculture. The fungi were cultured on a PDA medium for 5–7 days at ambient temperature. Then, plugs of active growing mycelium (diameter 0.4 cm) were inoculated into a 250 ml flask containing slightly modify submerged medium consisting of the following components (g/L): glucose, 40; yeast extract, 1.0; peptone, 0.5; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.5 as described in our previous work[7]. The pH was adjusted to 6.5, and then the flasks were incubated on a rotary shaker at 28°C and 150 g for 7 days.

(b) Exopolysaccharides extraction

Exopolysaccharides were extracted according to Kim *et al.*[18], the samples were redissolved in distilled water for determination of the total carbohydrate content, reducing power, total phenol content, protein content, and antioxidant activity.

Properties of Exopolysaccharides

(a) Determination of total carbohydrate content

The carbohydrate contents were determined with a phenol–sulphuric acid method according to Masuko *et al.*[24], the colour reaction was initiated by mixing 50 µl of exopolysaccharide solution with 150 µl of

concentrated sulphuric acid, followed immediately with 30 µl of 5% phenol, and the reaction mixture was kept at 90°C for 5min. After cooling to room temperature, the absorbance of the mixture was measured at 490 nm. The total carbohydrate content was calculated with D-glucose as standard.

(b) Determination of reducing sugar and protein

The reducing sugar was determined by the method of Miller (1959 briefly, 0.5 ml of 1 % 3, 5-dinitrosalicylic acid (DNS) was added to an aliquot of exopolysaccharide (20–500 µl) and the volume adjusted to 5ml with distilled water. After shaking, the mixture was heated in boiling water for 5min and cooled to room temperature; 2.5 ml of distilled water were added to the mixture. The absorbance was measured at 540 nm, and the total reducing sugar was calculated with D-glucose as a standard reducing sugar. Total polysaccharide was the subtraction of reducing sugar from the total carbohydrate.

Proteins were estimated by the Folin-Ciocalteau phenol reagent method[22] using bovine serum albumin (Sigma) as a standard.

(c) Preparation of cloudy apple juice and storage conditions

Apples were washed with running tap water, dried under fan and then cut into four pieces with the help of stainless steel knife. Apple juice was extracted using a household table top juice extractor (Multipress automatic Braun MP80, Kronberg, Germany) and filtered through sterilized double layered cheese cloth to remove impurities and coarse particles. To avoid undesirable enzymatic browning, 1g/L ascorbic acid was added to the pressed juice. Then, the samples were ûltered through a 4-layer cheese cloth and poured into beakers containing exopolysaccharides with different concentrations. The sample without adding exopolysacchrides considered as control. The juice treatments and the control were stored at 4°C (refrigerator) for two weeks and samples were taken out at intervals to be assayed.

(d) Measurement of juice cloud stability

During the storage, aliquots (10 ml) of the stored juice were drawn from the upper portion of the bottles. The cloud stability of the juice was determined before and after centrifugation at 4200 xg for 15 min, respectively in a 1 cm path cuvette cell as absorbance at 660 nm[36], using UV-Vis Shimadzu Spectrophotometer (UV-1601 PC, Japan).

(e) Analysis of total phenol content and phenolic degradation

The total phenol content and phenolic degradation of the studied samples were estimated by the Folin-Ciocalteu colourimetric method, based on the procedure described by Ibrahim *et al*.[17].

Antioxidant activity assays

(a) Scavenging of 1,1'-diphenyl-2-picrylhydrazyl (DPPH) radicals assay

The DPPH radical-scavenging activity of juices was determined according to the method of[37]. The radical-scavenging activity of the samples (antioxidant activity) was expressed as percent inhibition of DPPH radical as following:

$$\% \text{ Inhibition} = [(A_{control} - A_{treatment}/=A_{control})] \text{ X } 100$$

where: $A_{control}$: is the absorbance of the control; $A_{treatment}$: is the absorbance of the treatments. Butylated hydroxyl anisol (BHA), *tert*-Butylated hydroxyl qunione (TBHQ) were used as reference compounds.

(b) β–Carotene-linoleic acid assay

β-Carotene bleaching assay was carried out according to the method developed by Wettasinghe & Shahidi[34]. one milliliter of β-carotene solution (0.2 mg/ml chloroform) was pipetted into a round-bottom flask (50 ml) containing 0.02 ml of linoleic acid and 0.2 ml of 100% Tween 20. The mixture was then evaporated at 40°C for 10 min using a rotary evaporator (BUCHI, Germany) to remove chloroform. After evaporation, the mixture was immediately diluted with 100 ml of distilled water. The distilled water was added slowly to the mixture with vigorous agitation to form an emulsion. Five ml aliquots of the emulsion were transferred into different test tubes containing 0.2 ml of samples in 80% methanol at 1 mg/ml. The mixture was then gently mixed and placed in a water bath at 50 °C for 2 h. Absorbance of the sample was measured every 30 min for 2 h at 470 nm using UV-Vis Shimadzu Spectrophotometer (UV-1601 PC, Japan). Blank solution was prepared, containing the same concentration of sample without β-carotene. All determinations were performed in triplicate. The total antioxidant activity was calculated based on

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the following equation:

$$AA = [1-(A_{S(0)}-A_{S(120)})/(A_{b(0)}-A_{b(120)})] \times 100$$

where: AA: Antioxidant activity

Where, $A_{S(0)}$ is absorbance of sample at 0 min., $A_{S(120)}$ is absorbance of sample at 120 min., $A_{b(0)}$: is absorbance of blank at 0 min., $A_{b(120)}$: is absorbance of blank at 120 min.

Isolation and analysis of headspace volatiles

The volatiles in headspace of treatments under investigation were isolated using a dynamic headspace system. The samples were purged for ~ 3 h with nitrogen gas (grade of N_2 > 99.99%). The headspace volatiles were swept into cold traps containing diethyl ether and pentane (1:1, V/V) and hold at 10 °C. The solvents containing the volatiles were dried over anhydrous sodium sulphate overnight. The volatiles were obtained by evaporation of the solvents under reduced pressure.

Gas Chromatography analysis

Gas chromatography (GC) analysis was performed using Perkin-Elmer Autosystem equipped with ûame ionisation detector (FID). A fused silica capillary column DB-5 (60 m X 0.32 mm i.d) was used. The oven temperature was maintained initially at 50 °C for 10 min and then programmed from 50 to 180 °C at a rate of 3°C/ min. Helium was used as the carrier gas at a fow rate of 1.0 ml/min. The injector and detector temperatures were 220 and 250 °C, respectively. The retention indices (Kovats index) of the separated volatile components were calculated with hydrocarbons (C_6–C_{22}) as references.

Gas chromatographic–mass spectrometric analysis

The analysis was carried out using a coupled gas chromatography Hewlett–Packard (5890)/mass spectrometry Hewlett–Packard-MS (5970). The ionization voltage was 70 eV, mass range m/z 39–400 amu. The GC condition was carried out as mentioned above. The isolated peaks were identified by comparison of mass spectra of the target compounds with those of the National Institute of Standards and Technology (NIST) library and verified by the retention indices of pure standard compounds identified by matching with data from the library of mass spectra and compared with those of authentic compounds and published data[1].

Measurement of the polyphenolic compounds in apple juices by HPLC

Briefly, the extraction was performed with a 1:1 dilution of apple juice with HPLC grade methanol. Briefly, samples were injected onto a RP-HPLC column Zorbax 300SB C_{18} column (4.5 X 250 mm) (Agilent 1100 Technologies, USA) and eluted with a flow rate of 1 ml min^{-1} and a mobile phase containing: (A) acetic acid/water (2.5%) and (B) acetonitrile following a gradient: from 0 to 10 min, 0% B, from 10 to 40, 10% B, from 40 to 70, 40% B, up to 72, 100% B. The identification of the

TABLE 1 : The yield of total carbohydrates, reducing sugars, Exo-polysaccharides, protein, total phenol content and antioxidant activity of the six *Penicillium* species

Penicillium species	Total carbohydratesg/L	Reducing Sugars g/L	Exo-polysaccharides g/L	Protein mg/g	Total phenol content mg GAE/g	Antioxidant activity (%)	
						DPPH°	β-carotene
Eupenicillium ochrosalmonium	2.04± 0.12[a]	0.04± 0.13[a]	2.0 ± 0.11	33.2 ± 0.15	34.8± 0.15[a]	64.4± 0.12[a]	60.3± 0.17[a]
Eup. pinetorum	3.48± 0.15	0.05± 0.17[b]	3.43 ± 0.13	31.5 ± 0.19[b]	35.6± 0.12[a]	72.3± 0.15[b]	65.6± 0.19[a]
Penicillium crustosum	1.53± 0.20	0.03± 0.18	1.5 ± 0.17[a]	31.4 ± 0.12[b]	44.2± 0.13	58.0± 0.16[c]	53.2± 0.14[b]
P. chrysogenum	2.83± 0.17[b]	0.04± 0.12[a]	2.79 ± 0.20[b]	34.3 ± 0.22	40.1± 0.17	56.3± 0.11[c]	50.1± 0.20[b]
P. megasporum	1.88± 0.13[a]	0.06± 0.16	1.82 ± 0.19[a]	30.2 ± 0.24[a]	31.3± 0.20	61.3± 0.14[a]	59.3± 0.21[a]
P. olsonii	2.98± 0.21[b]	0.05± 0.15[b]	2.93 ± 0.21[b]	30.4 ± 0.20[a]	42.1± 0.16	66.4± 0.18[a]	63.4± 0.22[a]

***: Values are expressed as (mean ± SD, n=3); The same letters within the same column are not significant (P ≤ 0.05)**

components in the sample was done by comparing their retention time and UV spectra with those of polyphenolic compound external standards Hoang *et al.*,[15]. Each compound was quantified by comparing its peak area against the standard obtained specifically for the reference solutions containing that compound.

Statistical analysis

Results were given as mean±SD of three independent determinations. One-way analysis of variance (ANOVA) and least signiûcant difference (LSD) were performed using SPSS.14 to determine any signiûcant difference among various treatments was used to compare the means. Differences were considered to be signiûcant at $P < 0.05$[31].

RESULTS AND DISCUSSION

Compositional analysis of crude polysaccharides

The results in TABLE 1 showed that all the experimental fungi produced variable amounts of exopolysacchrides, it was clarified that the highest total carbohydrate content and exopolysaccaharides were resulted from the isolates; *Eup. Pinetorum*, *P. olsonii* and *P. chrysogenum*, are 3.48, 2.98 and 2.83 g/L for total carbohydrates and 3.43, 2.93 and 2.79 g/L for exopolysaccharides, respectively. With regard to the estimation of reducing sugar, it was shown that the exopolysaccharides of *P. megasporum* and *P. crustosum* had the highest and the lowest amounts of reducing sugar. The total phenol contents in the exopolysaccharides were also investigated. It was shown that *P. crustosum* and *P. olsonii* have the highest total phenol content (44.2 and 42.1mg GAE/g of polysaccharides, respectively). Moreover, protein content of the three above mentioned isolates, *Eup. Pinetorum*, *P. olsonii* and *P. chrysogenum* was 31.5, 30.4 and 34.3 mg/g, respectively.

TABLE 2 : Changes in the cloud stability (T%) in apple juice during two weeks of storage at 4 °C

Storage time (days) / Usage level uL/ 100 mL	Zero time	2	4	8	14
Control	96.3±1.09*[a]	92.2±0.31	89.5±0.28	88.6±0.91	87.4±0.24
Eupenicillium ochrosalmonium					
150	96.8±0.88	93.4±0.16[a]	92.3±0.34[a]	91.1±0.24	90.7±0.49[a]
300	97.4±0.34[b]	94.5±0.29[b]	93.7±0.29[b]	92.8±0.19[a]	91.6±0.34[b]
450	98.3±0.46	95.8±0.95[c]	95.1±0.24[c]	93.3±0.12[b]	92.5±0.28
Eup. Pinetorum					
150	98.3±0.28	95.4±0.37	94.8±0.17	94.2±0.18	92.3±0.17
300	98.7±0.21	95.7±0.12[c]	94.4±0.12	93.8±0.61	92.7±0.24
450	99.2±0.19	96.3±0.16	95.2±0.31[c]	94.7±0.17	93.2±0.25
Penicillium crustosum					
150	96.7±0.34	93.4±0.94[a]	92.7±0.15	92.6±0.26	91.4±0.16[a]
300	97.4±0.27[b]	94.7±0.39[b]	93.3±0.28	92.8±0.61[a]	91.6±0.12[b]
450	97.8±0.22[c]	95.2±0.67	94.8±0.23	93.3±0.58[b]	92.9±0.51
P. chrysogenum					
150	97.2±1.17	94.4±1.03	93.3±0.37	92.7±0.74	92.1±0.46
300	97.8±0.97[c]	94.7±1.07	93.8±0.12[b]	92.9±0.15[a]	92.5±0.24
450	98.4±0.45	95.2±0.92	94.2±0.19	93.4±0.16[b]	92.8±0.16
P. megasporum					
150	96.5±0.14[a]	92.2±0.86	91.6±0.20	90.6±0.13	89.7±0.12
300	96.8±0.18	93.3±1.08[a]	92.7±0.27[a]	91.4±0.24	90.8±0.24[a]
450	97.3±0.16[b]	93.9±2.03	92.9±0.91	92.2±0.28	91.5±0.29[b]
P. olsonii					
150	97.5±2.04	94.7±0.89[b]	93.8±0.64[b]	92.3±0.64	91.7±0.31
300	97.8±0.17[c]	95.3±0.94	94.2±0.29	93.7±0.91	92.2±0.27
450	98.2±0.16	95.8±1.06[c]	94.9±0.37	94.1±0.14	93.3±0.25

***: Values are expressed as (mean ± SD, n=3); The same letter within the same column are not significant (P ≤ 0.05)**

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Our results in good agreement with Dong & Yao[9] who found that high yields of exopolysaccharides were extracted from both natural and cultured mycelia of *Cordyceps sinensis*. While, Chen *et al*.[6], reported high exopolysaccharide production by the mangrove endophytic fungus *Aspergillus* sp. Y16. On the other hand, the obtained results was not in accordance with Thetsrimuang *et al*.[32], who found that the exopolysaccharide of *Lentinus polychrous* mycelia had the lowest total carbohydrate, whereas the exopolysaccharide of dried fruit body had the highest value while, mycelium was found to give the highest yield of crude polysaccharide, but with the lowest content of total carbohydrate.

Antioxidant activities of fungal exopolysaccharides

It was clarified from TABLE 1 that *Eup. Pinetorum* possesses potent antioxidant activity followed by *P. olsonii* and *Eup. ochrosalmonium* with scavenging effect amounted to 72.3, 66.4 and 64 %, respectively on DPPH radicals and 65.6, 63.4 and 60.3 %, respectively on ²-carotene assay. The effect of antioxidants on DPPH radicals scavenging is due to their hydrogen-donating ability, while, The bleaching mechanism of ²-carotene is a free radical mediated phenomenon resulting from the formation of hydroperoxides from linoleic acid oxidation[27]. In the absence of antioxidant, ²-carotene will undergo rapid discoloration. The addition of the antioxidant containing extracts can protect the extent of ²-carotene orange colour by neutralizing the peroxide products which were formed from linoleic acid. Our results are in accordance to Li *et al*[20]. who found that the DPPH scavenging activity was increased with the exopolysaccharide concentration increasing produced from *Berkleasmium* sp.

Cloud stability

During the storage period of 14 days, some bottles were taken out of the cultured box and some juice was drawn from the upper portion of the bottles and assayed directly or assayed after being centrifugation and the obtained data are displayed in TABLE 2. The added exopolysaccharides showed increase in cloud stability with increasing the concentration. Under our studied concentration the highest stability was in treatment with *Eup. Pinetorum* (99.2%) at 450 ul/100 ml juice followed by *P. chrysogenum* (98.4%) while the lowest stability had obtained in the treatment with *P. megasporum* (97.3%) at the same level at zero time.

As the storage time prolonged from zero to 14 d, the cloud stability in all treatments decreased significantly, but minimal decrease was observed in *Eup. Pinetorum* (93.2%). Our results in good manner with Genovese & Lozano[12] who found that the greater stabilizing effect of CMC in cloudy apple juice was basically due to its electronegativity.

Also, Zhang *et al*.[38], also found that alginate-Na with negative charge had an effective action in preventing sedimentation in combined vegetable juice.

Phenolic degradation and phenolic compounds

The obtained data of phenolic degradation in all treatments as well as the individual phenolic compounds in treatment with *Eup. Pinetorum* –selected sample- were given in (TABLES 3 and 4).

In TABLE 3 results of phenolic degradation of cloudy apple juice treated with several exopolysacchrides during storage are presented and revealed that with prolong the storage time an increase in phenolic degradation had occurred. Generally, the decline of phenol content was in the range from 16.90 % (*Eup. Pinetorum*) to 33.4 % (*P. megasporum*) after two days of storage. Phenolic degradation measured in the investigated samples was in line with[30].

During the storage experiment of juices with and without exopolysaccharides added at 4 °C for two weeks, the phenolic degradation and chlorogenic acid became more important by prolonged storage time (TABLES 3 and 4). Since the polyphenol oxidase was inactivated, the decrease of polyphenols was caused by non-enzymatic reaction.

In a previous study Carbone *et al*.[5], studied the influence of genotype, tissue type and cold storage on bioactive compounds of different apple cultivars, where total phenol content was dramatically reduced after cold storage (flesh 50 %; peels 20 %). On the other hand, results by Burda *et al*.[4], showed that the concentration of the major phenolics, epicatechins, procyanidin B_2, and phloretin glycosides in apple flesh remained at a relatively constant level during the storage. Since

TABLE 3 : Phenolic degradation in cloudy apple juice stored at 4 °C after different treatments at three concentrations (g/100 ml)

Storage time (days) / Usage level uL/ 100 mL	2	4	8	14
Eupenicillium ochrosalmonium				
150	32.6±0.02*	35.3±0.09[a]	39.7±0.52	40.6±0.27[a]
300	31.4±0.18[a]	34.2±0.12[b]	38.5±0.19[a]	39.7±0.30
450	30.5±0.31[b]	34.1±0.91	36.4±0.31[b]	37.8±0.28[b]
Eup. Pinetorum				
150	19.5±0.14	22.8±0.62[c]	26.4±0.37	28.3±0.91[c]
300	17.4±0.12	19.6±0.35	20.7±0.16	24.5±0.84
450	16.9±0.05	18.2±0.17	19.3±0.18	21.8±0.57
Penicillium crustosum				
150	34.2±0.17	36.4±.61	38.9±0.19[a]	40.1±0.31[a]
300	31.2±0.21[a]	33.7±0.25[d]	36.7±0.28[b]	38.4±0.29
450	30.7±0.16[b]	31.4±0.37	35.3±0.29[c]	37.9±0.25[b]
P. chrysogenum				
150	31.9±0.08[c]	33.7±0.31	35.6±0.51	37.2±0.19
300	29.7±0.14	31.4±0.18	32.7±0.19	35.6±0.14
450	28.3±0.12	30.8±0.12	31.8±0.13	34.5±0.34
P. megasporum				
150	33.4±0.31	35.8±0.37[a]	37.6±0.82	38.2±0.61
300	31.7±0.28[c]	34.2±0.62[b]	36.8±0.74	37.9±0.18[b]
450	30.8±0.15[b]	33.9±0.24[d]	35.4±0.62[c]	36.5±0.16
P. olsonii				
150	23.7±0.11	25.6±0.09	29.8±0.56	30.4±0.54
300	22.8±0.02	23.4±0.15	26.7±0.38	29.8±0.19
450	20.6±0.31	22.9±0.38[c]	25.8±0.47	28.6±0.17[c]

***: Values are expressed as (mean ± SD, n=3); The same letter within the same column are not significant (P ≤ 0.05)**

TABLE 4 : Effect of *Eup. Pinetorum* on phenolic compound concentrations (mg/100 mL) of Cloudy apple juice stored for two weeks at 4 °C

Phenolic compound	Control		*Eup. pinetorum*	
	Fresh	Stored	Fresh	Stored
Gallic acid	0.06±0.002*	0.03±0.001	0.95±0.15	0.84±0.09
Ellagic acid	0.28±0.04	0.14±0.01	0.36±0.43	0.29±0.01
Qurecetin	0.07±0.001	0.06±0.003	0.14±0.07	0.11±0.007
Chlorogenic acid	12.9±0.14	6.72±0.21	13.72±0.16	12.96±0.18
Cinnamic acid	0.83±0.05	0.61±0.04	0.94±0.03	0.87±0.05
Pyrogallol	0.43±0.06	0.29±0.19	0.55±0.22	0.43±0.12
Synirgenic acid	1.14±0.04	0.87±0.13	1.34±0.18	1.29±0.17
Catechin	0.09±0.008	0.01±0.005	0.07±0.004	0.01±0.003
Ascorbic acid	1.37±0.19	0.74±0.08	1.38±0.12	0.25±0.04
P-Qumaric acid	0.76±0.03	0.56±0.017	0.85±0.05	0.78±0.17

***: Values are expressed as (mean ± SD, n=3)**

individual phenolic compounds have shown to vary in their browning rates, it is important to know that the concentration of individual phenol in apples changes during the storage.

The significant change of the phenolic degradation may be due to higher concentration of the individual

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phenolic compounds, such as chlorogenic acid, ascorbic acid and synirgenic acid. These compounds are well recognized as substrates of apple polyphenol oxidase and/or are involved in nonenzymatic coupled oxidation mechanisms[3].

A total of 10 phenolic compounds (TABLE 4) were identified by HPLC system in investigated treatments which were gallic acid, ellagic acid, Qurecetin, cholorgenic acid,cinnamic acid, pyrogallol, synigenic acid, catechin, ascorbic acid and *P*-qumaric acid.

The contents of chlorogenic acid as the major polyphenolic compound, representing 66.99-72.69% of the total polyphenols detected in stored control and stored treatment of *Eup. Pinetorum*, respectively. Other authors have reported high levels of this phenolic compound in apples[33].

Antioxidant properties

(a) Scavenging ability on DPPH° radicals

As is shown in TABLE 5, the cloudy apple juice treated with six exopolysaccharides of *Penicillium* sp. During storage for 14 days at 4 ^{0}C showed increase in antioxidant activity with increasing the applied concentrations. However reversible relationship had occurred with increase the storage time.

The order of studied exopolysaccharides in antioxidant activity was *Eup. Pinetorum* > *P. olsonii* > *P. chrysogenum* > *Eupenicillium ochrosalmonium* > *P. megasporum and Penicillium crustosum* had the lowest antioxidant activity at the same concentration 450 ul/100 ml. There is no significant ($P \leq 0.05$) between *Eup. Pinetorum* and BHA after two days of storage at 4 ^{o}C.

The complexity of exopolysaccharides molecular structure and chemical composition of polysaccharides

TABLE 5 : Antioxidant activity of cloudy apple juice treated with six exopolysacchrides during two weeks of storage at 4 °C as determined by DPPH° and β-Carotene assays

Storage time (days)	DPPH^0					β-Carotene				
Usage level uL/ 100 mL	Zero time	2	4	8	14	Zero time	2	4	8	14
Control	61.7±1.13*	55.3±0.64	51.8±0.61	49.5±0.27	48.2±0.29	58.7±0.81	56.3±0.64	49.8±1.06	47.2±0.79	45.9±0.72
Eupenicillium ochrosalmonium 150	85.4±0.28	79.2±0.31^a	71.6±0.28^a	69.4±0.13^a	66.8±0.11	64.2±0.79	61.9±0.82^a	59.7±0.28^a	57.2±0.84^a	55.4±0.34^a
300	86.7±0.37	81.6±0.22^b	77.5±0.19^b	72.3±0.27^b	69.4±0.16	66.8±0.72^a	62.4±0.94^b	60.8±0.31^b	59.4±0.15^b	56.8±0.28
450	88.3±1.16^a	82.4±0.14^c	79.3±0.24	74.9±0.26	71.5±0.17	67.3±0.64^b	63.5±0.37	61.4±0.39	60.7±0.34^c	57.2±0.46
Eup. Pinetorum 150	90.2±0.94^b	88.5±0.19^d	87.5±0.47	86.4±0.52	85.7±0.54	71.4±0.28	69.8±0.64	67.2±0.15^c	65.9±0.54^d	64.8±0.51
300	92.6±0.83	90.3±0.31	88.2±0.51^c	87.9±0.18	86.3±0.29	72.6±0.19	70.5±0.58	68.7±0.28	66.5±0.82	65.1±0.29
450	93.7±0.24	91.8±0.25	90.8±0.37	88.5±0.14^c	87.9±0.24	75.9±0.34	71.3±0.91	69.8±0.52	68.3±0.73^e	67.4±0.38
Penicillium crustosum 150	79.8±2.01	75.6±0.15	70.5±0.14	67.4±1.02	65.1±0.08	61.4±0.15	57.1±1.12	55.8±0.13	53.4±0.52	51.8±0.16
300	80.4±1.16	76.9±0.45	71.3±0.29^a	69.2±1.13^a	66.8±1.03	62.8±0.17	58.3±0.67	56.9±0.25	53.9±0.18	52.4±0.18
450	81.3±1.28	78.2±0.61	72.9±0.65	70.6±0.27	67.4±1.05	63.7±0.61	58.9±0.83	57.2±0.29	54.2±0.29	53.6±0.25
P. chrysogenum 150	86.4±0.31	82.7±0.82^c	80.5±0.38	77.6±1.02	75.8±0.28^a	65.8±0.28	63.5±0.95	60.8±1.02^b	59.4±0.61^b	58.7±0.83
300	88.2±0.18^a	83.6±0.91	81.4±0.22	78.4±0.84	76.9±0.29	67.9±0.81^b	65.4±1.16	61.3±0.55	59.7±0.25	59.6±0.97
450	89.7±0.29	84.5±0.37	81.9±0.11	79.3±0.75	77.5±0.24^b	68.2±0.63	66.1±0.39	62.7±0.24	60.3±0.19^c	60.3±1.03
P. megasporum 150	81.5±0.11	79.3±0.12^a	77.8±1.05^b	72.7±0.15^b	70.4±0.38	63.7±0.22	61.5±0.61^a	58.4±0.19	56.5±0.84	54.2±0.31
300	82.4±0.24	81.6±0.14^b	79.2±1.12	73.4±0.19	72.8±0.45	65.3±0.13	62.4±0.58^b	59.9±0.24^a	57.8±0.37^a	55.3±0.11^a
450	83.9±0.26	82.5±0.35	80.6±0.92	75.8±0.24	73.2±0.71	66.9±0.18^a	62.8±0.73	61.2±0.67	59.3±0.19^b	55.9±0.08
P. olsonii 150	88.4±0.17^a	84.5±1.12	81.7±0.21	76.4±0.31	74.8±1.21	68.5±0.12	66.2±0.26	65.7±0.43	64.5±0.11	63.2±0.37
300	89.6±1.14	86.3±2.07	82.5±0.16	78.9±0.26	75.2±1.06^a	69.4±0.37	67.8±0.37	66.1±0.28	64.8±0.34	63.9±0.88
450	90.5±1.06^b	87.9±0.21	83.2±0.19	79.1±0.18	77.5±1.14^b	70.3±0.82	68.9±0.29	67.3±0.39^c	65.3±0.29^d	64.2±0.92
BHA 200 ppm	89.1±0.05	89.1±0.04^d	88.7±0.02^c	88.6±0.03^c	88.5±0.06	68.4±0.06	68.3±0.02	68.3±0.05	68.2±0.04^e	68.1±0.03
TBHQ 200 ppm	94.3±0.01	94.3±0.06	94.2±0.05	94.1±0.08	94.1±0.02	76.2±0.04	76.2±0.01	76.1±0.03	76.1±0.01	76.1±0.02

***: Values are expressed as (mean ± SD, n=3); The same letter within the same column are not significant ($P \leq 0.05$)**

TABLE 6 : Effect of exopolysaccharides produced by *Eup. pinetorum* and storage on volatile compounds identified in headspace of cloudy apple juice (CAJ)

Volatile compound	RIs[a]	Control		*Eup. pinetorum*		Odour description[c]	Identification method[d]
		Fresh	Stored	Fresh	Stored		
Esters							
Ethyl ethanoate	596	2.34[b]	1.26	1.85	1.73		MS, RI, St
Ethyl acetate	642	15.29	12.94	16.32	14.98		MS, RI, St
Propyl acetate	698	2.53	0.75	2.81	1.84	Strong-sweet	MS, RI, St
Ethyl propanoate	745	1.83	0.91	1.96	1.76	Pungent	MS, RI
Methyl butanoate	753	1.37	0.95	1.52	1.48	Sweet, apple-like	MS, RI
Isobutyl acetate	769	0.53	0.62	1.13	1.10		MS, RI, St
Butyl acetate	828	5.92	3.71	6.31	5.78		MS, RI
Ethyl butanoate	849	7.48	3.69	7.59	6.41	Apple-like, fruity	MS, RI, St
Ethyl-2-methyl butanoate	875	2.75	18.24	27.51	25.86		MS, RI
2-Methylbutyl acetate	885	2.21	0.18	0.63	0.58	Sweet, apple-like	MS, RI
Propyl butanoate	897	0.47	0.23	0.57	0.44		MS, RI, St
Ethyl pentanoate	956	1.19	0.12	0.84	0.71		MS, RI, St
Butyl butanoate	997	3.52	2.89	4.61	3.83		MS, RI
Ethyl hexanoate	1001	0.65	0.52	0.75	0.39	Rotten apple	MS, RI, St
Hexyl acetate	1015	1.18	0.74	2.57	1.16		MS, RI, St
Hexyl butanoate	1187	1.94	0.62	1.32	1.18		MS, RI
Alcohols							
Ethanol	615	15.67	24.29	7.81	14.53	Green, grass-like	MS, RI, St
1-Butanol	693	8.51	13.45	5.47	8.38	Sweet	MS, RI, St
Aldehydes							
3-Methyl butanal	751	1.28	0.63	2.17	2.31	Sweet, apple-like	MS, RI
Hexanal	773	19.61	5.67	1.19	1.43		MS, RI, St
Ketones							
1-Penten-3-one	742	0.42	0.31	1.75	1.49		MS, RI
Monoterpene hydrocarbons							
D-Limonene	1031	1.29	1.65	1.32	1.27	Cheesy	MS, RI, St

[a]: retention indices; [b]: Values are expressed as relative area percentage to the total identified volatile compounds. [c]: Odour descriptions cited from Aaby et al. (2002),Plotto and Mc-Daniel (2001) and Fukami et al.(2002). d:b:Compounds identified by GC-MS(MS) and/or by comparison of MS and RI of standard compound (St) run under similar conditions

fractions isolated from medicinal fungi makes it difficult to elucidate their structure and activity relationships. It has been suggested that the antioxidant capacity of exopolysaccharides molecules may be ascribed to their hydroxyl group, which donates electrons to reduce the radicals to a more stable form or reacts with the free radicals to terminate the radical chain reaction. In the present study a significant correlation between antioxidant activity as determined by DPPH and β-carotene assays and phenolic degradation had observed and these results are in agreement with[7].

To the best of our knowledge this study is the first trial to investigate the antioxidant activity of exopolysaccharides in real food system.

Moreover, the antioxidant capacity of polysaccharides molecules also depends strongly on the type and organization of sugar monomers, the linkage pattern of the main chain (α or β) and the branching configuration[21].

(b) Scavenging ability on β-carotene

The relative ability of the exopolysaccharides to act

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as antioxidants was investigated through *in vitro* models such as the β-carotene–linoleate model system. The antioxidant activity of the added exopolysaccharides was assayed at 150, 300, 450, uL/100 ml cloudy apple juice and was compared with BHA and TBHQ (TABLE 5). The antioxidant activity of the exopolysaccharides was found to increase with an increase in its concentration. *Eup. Pinetorum* showed nonsignificant activity compared to BHA after 8 days of storage (TABLE 5).

In the present study, it was observed that the polysaccharides hindered the extent of β-carotene bleaching by neutralizing the linoleate free radical and other free radicals formed in the system. Antioxidant activity of antioxidants might be attributed to some mechanisms such as preventing chain initiation, binding transition metal ion, decomposing peroxides, preventing continued hydrogen abstraction, reducing ability and scavenging free radical[14].

(c) Analysis of volatile

Volatile compounds in the fresh and stored control cloudy apple juice as well as treated by *Eup. Pinetorum* at zero time and after two weeks of storage at 4 °C and their KIs are listed in TABLE 6. Twenty-two volatiles, namely 16 esters, 2 alcohols, 2 aldehydes, 1 ketone and 1 monoterpene hydrocarbons were identified in the investigated samples.

Cloudy apple juice is mainly composed of esters (51.2%), alcohols (24.18%) and aldehydes (20.89%) in the fresh control sample. The major alcoholss were identified as ethanol and butanol in the control and treated sample with *Eup. Pinetorum.*

Esters made up to 52.1%, 48.37%, 78.29% and 69.23% of the total volatile compounds in fresh control, stored control, treated by *Eup. Pinetorum* at the beginning of storage and stored one, respectively. The major compounds were ethyl acetate, ethyl butanoate, butyl acetate,ethyl-2-methyl butanoate, ethyl ethanoat and propyl acetate (TABLE 6). Many researchers have reported that esters play an important role in the flavour of apples[10], they also reported that esters make up to 87% as predominant volatile compounds according to the condition of storage. Ethyl butanoate was suggested to be the important odorant in 40 apple cultivars[8].

Alcohols were the predominant chemical group of the volatile compounds in cloudy anna apple juice, with a proportion of up to 24.18% and 37.74% in fresh and stored control, respectively. The major compounds belonging to alcohol were ethanol and butanol. Previous reports showed that these alcohols are the most dominant compounds in apple[10]. he esters and alcohols which are the products of fatty acid metabolization were the major groups 44% and 41% of totals volatiles in the apple juice, respectively[11].

Aldehydes were the highest proportion of the volatile compounds in anna cloudy apple juice accounting for 20.89% and 3.36% of the total content in fresh control and treatment of *Eup. Pinetorum,* respectively. While, hexanal was the major aldehyde in fresh and stored control, 3-methyl butanal was identified as major compounds the sample treated by *Eup. Pinetorum* (TABLE 6). These results confirmed by Komthong *et al*.[19], who reported that hexanal is the most potent aromatic aldehyde with green/grassy odour in apple. Due to the low threshold value and the relatively high concentration, hexanal was regarded as the main contributor to grassy odour in the headspace gas. In addition to anna apple, hexanal was described to be the most potent odourant in other apples and unripe fruits. Hexanal was the most potent odorant in aldehyde group. Due to the low threshold value and the relatively high concentration, hexanal was regarded as the main contributor to grassy odor in the headspace gas. In addition to anna apple, hexanal was described to be the most potent odorant in other apples and unripe fruits[25].

CONCLUSION

The obtained results show that, adding of exopolysaccharides of six *Penicillium* species on the quality parameters of cloudy apple juice was stable for extended periods of storage. It was indicated that exo-polysaccharides from *Eup. Pinetorum* possessed considerable efficiency to prevent phenolic degradation during storage and significant radical scavenging activity on stable DPPH radicals and B-carotene assays. Exo-polysaccharides from *Eup. Pinetorum* should be regarded as a valuable product and has potential as a value-added ingredient for functional foods.

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