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Biological Valorization of the Pod of Pea *Pisum sativum* L.

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ABSTRACT

Background: This study was conducted to evaluate the antioxidant activity of the pod pea essential oil and to determine the potential use of this vegetable waste as a medium for lactic acid production by *Lactobacillus plantarum*.

Methods: Antioxidant activity of the essential oil was determined by DPPH, iron reducing and lipid peroxidation methods. Pod pea syrup was obtained by addition of two litres of water at 80-85°C to 800 g of pod pea for 2 h. Other syrups were obtained under the same conditions by partially substituting 40 g of pod pea for the same amount of glucose, date and carob.

Result: At a concentration of 1000 µg mL^{-1}, the pod pea essential oil demonstrated good DPPH scavenging activity 89.30±1.4% as well as significant iron reducing. This oil is effective at inhibiting the lipid peroxidation. An important quantity of biomass (optical density of 1.55) and lactic acid 20.4 g L^{-1} were produced. The replacement of 20 g L^{-1} pod pea by the same quantity of glucose, date and carob improves the production of lactic acid.

Key words: Antioxidant, Essential oil, Fermentation, *Lactobacillus plantarum*, Pod pea.

INTRODUCTION

Oxidative stress (OS) has been one of the main interests of the scientific community for centuries. During the cell metabolism mechanism, a series of reactive oxygen species (ROS) are synthesized, such as superoxide anion, hydrogen peroxide and hydroxyl radicals (Rana and Dahiya, 2019). The imbalance between antioxidant defense system and ROS may lead to patho-biochemical mechanisms caused by the development of different degenerative diseases (Kaur *et al.* 2019). Antioxidants are vital agents that possess the ability to protect the body from all damages caused by free radical induced OS and retard the advancement of several chronic diseases (Kaur *et al.* 2019; Razni *et al.* 2019). Recently, the synthetic antioxidant agents are restricted because they are suspected to have some toxic effects. Hence, the studies on natural antioxidants have gained intensively greater importance. Legumes belonging to the *Leguminosae* family are widely consumed all over the world due to its high nutritive quality and low cost (Singh *et al.* 2004). The green pea (*Pisum sativum* L.) is the most significant legume. Field *Pisum sativum* is the principal pulse crop in Algeria. Composition and nutritional quality are important to consumers of both food and animal feed, but there is little information available on the utilization of pods. Knowledge of the biological activities of pod pea is beneficial for the valorization of this vegetable waste. Recently, researchers have begun to evaluate the total quantity of polyphenol and the biological activities of seed peas and peel pea extracts (Hadrich *et al.* 2014). However, the antioxidant capacity of pod pea essential oil (EO) was not determined.

The interest in using agricultural waste in biotechnological processes for the production of added value products such as lactic acid has been increasing worldwide in recent years. There is a large amount of agricultural residues and agro-products that could be used as an alternative source of carbon and energy due to their high sugar and organic matter content, which can serve as a favorable medium for the growth of lactic acid bacteria. However, until now, no studies have been carried out on the potential biotechnological valorization of pod pea for the production of added value products. Lactic acid fermentation is a product inhibited process; high acid tolerance is a distinctive feature of *Lactobacillus plantarum* (Sharma and Mishra, 2014). Therefore, this strain was used in the second part of this work for the production of lactic acid. However, pods of pea contain lower quantities of fermentable sugar. The addition of glucose or fruits rich in sugar, such as dates and carob, improves the performance of fermentation. The current study was conducted to determine the antioxidant activity of the essential oil extracted from the pod peas and to evaluate the potential use of pod peas as a medium for the production of lactic acid by *L. plantarum*.

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MATERIALS AND METHODS

Plant material

All experiments were carried in Bioconversion Laboratory, Microbiology Engineering and Health Saftey, (Mascara

University, Algeria). Commercially pea was purchased from the local market (Mascara, North-West of Algeria) in March 2018. The species was identified by a botanist at SNV faculty, Mascara University. The pod pea was washed with tap water to remove all impurities and then with distilled water. The samples were dried in darkness at room temperature and chopped into small particles.

Antioxidant activity

Isolation of essential oil

100 g of dried pod peas in 500 mL of distilled water were submitted to hydro-distillation for 3 h, using a Clevenger-type apparatus (ST15 OSA, Staffordshire, UK) until total recovery of EO. The obtained oil was dried over anhydrous sodium sulfate and stored at 4°C until tested in an opaque glass bottle sealed. The oil yield was evaluated by gravimetric method and expressed in terms of % w/w (ratio between the weight of the extracted oil and the weight of the tested sample). The purity of oil was verified by measurement of physicochemical parameters according to the European Pharmacopoeia (2000).

DPPH free radical-scavenging activity

The ability of the oil to scavenge free radical DPPH was carried out by using the method as described by Kirby and Schmidt (1997) with some modifications. The tested sample was prepared in methanol to achieve the concentration of 1 mg mL^{-1}. Binary dilutions were made to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.62, 7.81, 3.90, 1.99 and 0.97 µg mL^{-1}. A volume of 50 µL of each of these solutions was added to 1950 µL of methanol solution of DPPH (6.10^{-5} M) as free radical source. The mixtures were stirred for 30 seconds and then incubated in the dark for 30 min at room temperature. The absorbance was measured using UV/Vis spectrophotometer model Hitachi 4-2000 at 517 nm against pure methanol. Catechin and ascorbic acid were used as positive control. Lower absorbance indicates higher free radical-scavenging ability. The inhibition level of DPPH was evaluated using the equation:

$$\text{DPPH radical sacvenging activity level, \%} = \frac{\text{A control- A sample}}{\text{A control}} \times 100 \quad \text{....(1)}$$

Where:

Absorbance of the control containing all reagents except the oil and the sample (presence of the EO). IC50 (concentration of tested antioxidant that inhibits 50% of the DPPH radicals present in the reaction medium) was calculated from the % inhibition versus concentration plot, using a non-linear regression algorithm.

Ferric-reducing power

The ferric reducing power was evaluated by using the potassium ferricyanide-ferric chloride assay as described by Yildirim *et al.* (2001). 1 mL of tested sample at different concentrations was mixed with 2.5 mL of potassium ferricyanide solution $K_3Fe(CN)_6$, 1% and 2.5 mL of 0.2 M phosphate buffer pH 6.6. After incubation for 20 min at 50°C, 2.5 mL of trichloroacetic acid (10%) was added and the reaction mixture was centrifuged for 10 min at 3000 rpm (Sigma labrzentrifugen D-37620 Osterode am Harz, Germany). A volume of 2.5 mL of the supernatant from each mixture was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride solution (0.1%) prepared freshly in distilled water. After 20 min of reaction time at 35°C, the absorbance was recorded at 700 nm against a blank that contains all reagents except the EO solutions and ferric chloride. The control was performed by using different concentrations of catechin and ascorbic acid. Higher absorbance of the reaction mixture indicates higher reducing power. The concentration providing 0.5 of absorbance (IC_{50}) was determined by plotting absorbance at 700 nm against the sample concentration.

Liver lipid peroxidation assay

The lipid peroxidation level in the liver tissues was determined using the thiobarbituric acid reactive substances method as reported by Tatiya and Saluja (2010). The mixture contained 0.5 mL of homogenate 10%, 1 mL of KCl (0.15 M) and 0.5 mL of different dilutions of the tested antioxidant agent. The lipid peroxidation was initiated by the addition of 100 µL of ferric chloride 1 mM. After incubation for 30 min at 37°C, the reaction was stopped by addition of 2 mL of iced HCl 0.25 N containing 15% TCA trichloroacetic acid (tissue homogenate was deprotenized by TAC), 0.38% thiobarbituric acid and 0.2 mL of Butylated Hydroxyl Toluene (0.05%). The mixture was heated for 60 min at 80°C, cooled and centrifuged at 6900 rpm for 15 min. The absorbance of the supernatant was recorded at 532 nm against a blank containing all regents except the liver homogenate and tested antioxidant agents. Identical experiments were prepared to determine the normal (without tested antioxidant and $FeCl_3$) and the level of lipid peroxidation in the tissues (with $FeCl_3$ and without tested samples). The anti lipid peroxidation level (% ALP) was evaluated by the formula:

$$\%\ \text{ALP} = \frac{\text{A of } FeCl_3\text{- A of sample}}{\text{A of } FeCl_3\text{- A of normal}} \times 100 \quad \text{....(2)}$$

Fermentation of pod pea syrup

Biological material

Lactobacillus plantarum strain was graciously supplied by the Laboratory of Reaction and Process Engineering (LRPE, ENSAIA, Nancy-France). It was grown on MRS medium with 10% glycerol and kept at -20°C. The reactivation step was achieved after two successive transplantations at 37°C for 2h in liquid MRS broth. The pH was adjusted to 6.5 prior to sterilization at 108°C for 15 min.

Vegetable material

Pod pea was used as a natural medium for the growth of *L. plantarum.* Carob (*Ceratonia siliqua* L.) and date (*Phoenix*

dactylifera L.) variety *HMIRA* at *tmar* stage were respectively harvested in September 2018 from the region of Elbordj (Mascara, Algeria) and the region of Adrar (South-West of Algeria). The choice of these vegetable materials was based on their availability, low grade, important nutritive value especially for the fermentable sugars such as glucose and fructose (for date and carob) and low food and biotechnological usage (for pod pea). The pods without seeds of pea and carob and the fruit of date were washed with distilled water to remove all impurities, dried at room temperature to facilitate the grinding operation, shopped into small pieces to increase the surface of diffusion, then grinded and sieved to obtain flour from the vegetable waste.

Syrups extraction

Four vegetable syrups were extracted: Pod pea syrup (PPS) prepared at concentration of 40%, PPS with 20 g L^{-1} of glucose (PPSg), with 20 g L^{-1} of date (PPSd) and with 20 g L^{-1} of carob (PPSc). PPS was added by glucose, date or carob to improve its carbohydrate content. For preparation of the PPS, two litres of water at 80-85°C were added to 800 g of pod pea during 2h, then homogenized with a mixer Ultra-Turrax T25 (IKA-Werke GmbH, Germany) and filtered through a cloth (Turhan *et al.* 2010). The extracted syrup was centrifuged for 10 min at 15000 rpm and left standing for 48 h at room temperature to precipitate large quantities of insoluble substances and then filtered. Other syrups were made by the addition of 40 g of glucose, date or carob to 760 g of pod pea and then mixed with two litres of hot water and prepared under the same conditions. The final syrups went through the non-deposit phase of Watman filter paper (N°45).

Fermentation conditions and analytical methods

All cultures were carried out in a 2 L jar fermenter (Applikon Biocontroller ADI 1030) with an initial volume of 2 L. The pH was adjusted to 6.5 prior to sterilization at 120°C for 20 min. The inoculum culture was incubated at 37°C for 12h under agitation of 200 rpm before being transferred to the fermenter in a 10% ratio. The culture pH was maintained at 6.5 by automatic basic regulation with NH_4OH solution during the time of fermentation. All batch fermentations were undertaken by culture of *L. plantarum* on four extracted syrups (PPS, PPSg, PPSd, PPSc). The samples were withdrawn at desired time and frozen for further analysis. The biomass was determined by measurement of the optical density (OD) at 600 nm. For each sample, the cell suspension was centrifuged at 7000 rpm for 5 min at 4°C, diluted and filtered. Residual sugar was evaluated at 480 nm by Dubois method (Dubois *et al.* 1956). Standards were prepared with different concentrations of glucose solutions. Lactic acid concentration was evaluated by Multi parameter Medical Analyzer. The enzymatic kit used for the dosage was the PAP ref-61 192.

The kinetic parameters of the batch fermentations were evaluated by the calculation of the specific rate of growth μ in h^{-1}, of sugar consumption Qs in g g^{-1} h^{-1} and of lactic acid production QL.a. in g g^{-1} h^{-1} (Bimbenet and Loncin, 1995).

$$\mu = \frac{\frac{dX}{dt}}{X} \quad ....(3)$$

$$Qs = -\frac{\frac{dS}{dt}}{X} \quad ....(4)$$

$$QL.\,a. = \frac{\frac{dX}{dt}}{X} \quad ....(5)$$

The productivity represents the amount of lactic acid formed per liter per hour. The yield of growth (Yx/s in g g^{-1}) and of lactic acid formation (YL.a/s in g g^{-1}) were determined from the slopes of the plotted linear curve (X-X0) versus (S0-S) for growth and P-P0 versus (S0-S) for lactic acid production. Where X, S, P represents respectively the amount of biomass, residual sugar and lactic acid at time (t) and X0, S0, P0 represent the amount of these substances at time 0.

Data analysis

All determinations were conducted in triplicates and the results for each measured parameter were expressed as mean±SD. Data were statistically determined by analysis of variance ANOVA using the confidence level (*P<0.05*) using SPSS statistics software 8.1.

RESULTS AND DISCUSSION

The EO was successfully extracted at a small yield of 0.42±0.01%, this value was lower compared to other plants. This oil was characterized by a liquid limpid aspect, mobile, volatile, with yellow tint, strong and fresh smell. The physical analysis (density at 20°C 0.84±0.02 g mL^{-1}, relative density 0.97±0.02 g g^{-1}, pH 5.4±0.1, refractive index at 20°C 1.612±0.003, miscibility with ethanol at 96% (1/5, v/v), rotatory power+2.6°±0.1) and chemical indices (acid 6.73±0.01 mg KOH g EO^{-1}, saponification 134.64±0.02, carbonyl 81.34±0.02, ester 127.91±0.01 mg KOH g EO^{-1} at 20°C, peroxide 10±0.2 meq O_2Kg EO^{-1} and iodine 1.3±0.1) indicates the good quality of the extracted oil according to the European Pharmacopoeia (2000).

Antioxidant activity

The ability of the tested samples to scavenger the free radical DPPH was presented in Fig 1-A. The results reveal that the antioxidant capacity directly depends on the concentrations used. At a concentration of 1 mg mL^{-1}, a maximal scavenging level of DPPH was obtained by ascorbic acid at 96.23±1.20% (IC_{50} of 32.5±0.5 µg mL^{-1}), followed by catechin at 94.5±1.4% (20±0.2) and then EO 89.3±1.4% (143±0.3). The IC_{50} result of EO was lower compared to those cited by Hadrich *et al.* (2014) obtained from the different fractions of the pea: Water extract (>1000), methanol fraction (650±0.05) and ethyl acetate extract (350±0.02 µg mL^{-1}). This oil displayed a

strong antioxidant capacity due to its richness in different bioactive compounds. Plants of *Pisum sativum* synthesize an important quantity of Kaempferol and quercetin under light treated pea (Bottomley *et al.* 1966). According to Han and Baik (2008), the total phenolic content of green pea seeds is 1.2 mg g^{-1}. These flavonoids are mainly responsible for the antioxidant activity of peas.

As shown in Fig 1-B, an important activity for reducing iron was obtained by pod pea EO (OD of 1.5±0.3) at concentration of 1 mg mL^{-1}. This value was much higher than the OD obtained from different extracts of peel pea cited by Hadrich *et al.* (2014). The reducing power of EO was lower than the capacities revealed by catechin (2.660±0.016) and ascorbic acid (2.07±0.03) at same concentration. Similar values of IC_{50} were obtained by catechin 53±0.3 µg mL^{-1} and ascorbic acid 58.3±0.4 µg mL^{-1}. The lower value of IC_{50} displayed by the EO 135±0.2 µg mL^{-1} indicates it strong reducing power. A positive correlation exists between antioxidant activity and reducing power of some bioactive compounds. Reductones plays an important role for the reducing power of EO. The antioxidant capacity of reductones is based on the breaking of the free radical chain by donating a hydrogen atom (Krishnamoorthy *et al.* 2011).

Presented result (Fig 1-C) indicates the capacity of tested oil to protect against lipid peroxidation. This power revealed by the anti-lipid peroxidation level of 73±1% at 1 mg mL^{-1}. This value was close to those found by catechin 87.65±2% and ascorbic acid 88.39±2% at same concentration. The generation of malondialdehyde and associated substances which react with the thiobabiturique acid are inhibited by pulp oil (Tatiya and Saluja, 2010). This indicates important capacity of inhibiting lipid peroxidation of EO. Phenolic compounds are good nucleophiles and have the ability to act as radical scavengers and inhibit lipid peroxidation, acting as breakers generated through lipid peroxidation.

Fermentation of pod pea

As shown in Fig 2, for PPS, *L. plantarum* starts with an initial OD of 0.2 and reaches a final value of 1.55 after 72h of culture. During this fermentation, the strain consumes 33.5 g L^{-1} of sugar (Fig 3, Table 1). The sugar consumed was used as a carbon source for cell growth (biomass formed 1.35) and for production of 20.4 g L^{-1} of lactic acid (Fig 4). This fermentation was characterized by a long latency phase 34 h which corresponds to the adaptation of the strain to this new medium. The substitution of 20 g L^{-1} of pod pea by same concentration of glucose, date and carob promotes cell growth and the formation of lactic acid. The addition of glucose seems to have a beneficial effect on the growth and production of lactic acid. The cell adaptation phase was reduced from 34 h to 20 h. The quantity of lactic acid formed was 30.4 g L^{-1} and the amount of sugar consumed during this fermentation was 62 g L^{-1}. The fruits of dates and carob pods are an excellent source of sugar. The addition of these two vegetable materials to the pea syrup improves it carbohydrate content. This explains the important amount

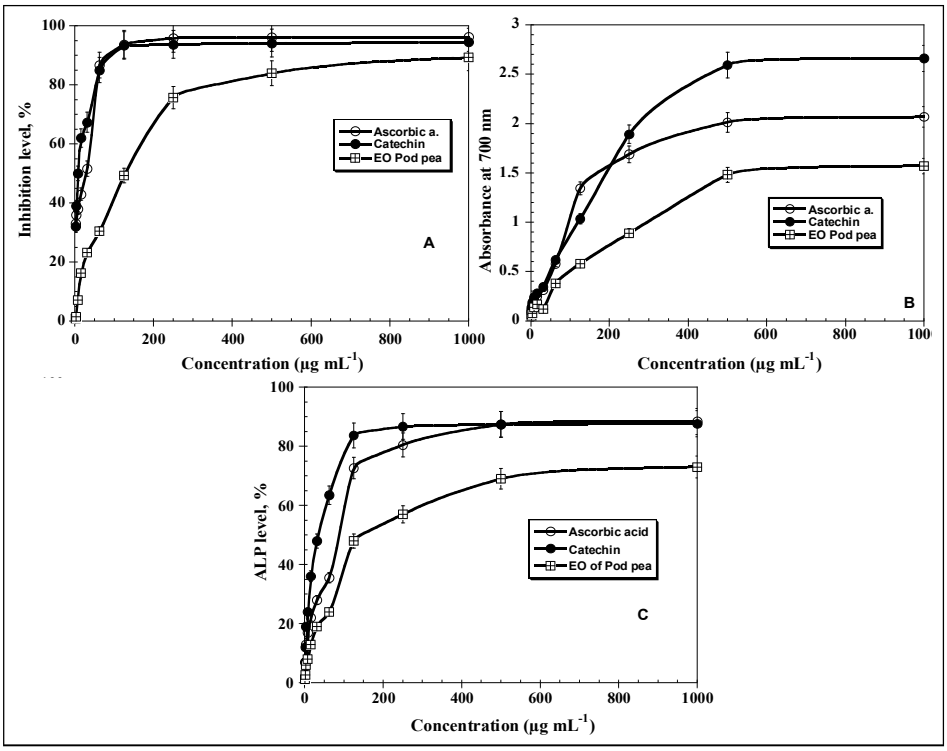

Fig 1: Antioxidant activity of ascorbic acid, catechin and oil of pod pea. Values represent Mean±SD; n=3; Confidence level $p \leq 0.05$ (A. DPPH method, B. ferric reducing power, C. lipid peroxidation assay).

of biomass (1.5 for PSPd and 1.48 for PSPc) and lactic acid (28.76 for PSPd and 23.5 for PSPc) obtained. *L. plantarum* ferment hexose carbohydrates to lactic acid through glycolysis due to the presence of a constitutive fructose-1,6-diphosphate aldolase, while ferment pentoses to lactic and acetic acids through the inducible 6-phosphogluconate/ phosphoketolase pathway (Axelsson, 1998). Chengjie *et al.* (2016), report that amino acids and nucleotide precursors are essential nutrient requirements for *Lactobacillus plantarum* fermenting milk. *L. plantarum* has amino acid auxotrophs and also vitamin auxotrophs. Mineral salts were classified as stimulators rather than essential nutrients for this strain. Six key amino acids (Ile, Leu, Val, Tyr, Met and Phe) and at least one purine (adenine or guanine) were specifically identified as minimal nutrients for *L. plantarum*. Moreover, the addition of dates or carob is necessary to the medium for improving the growth of the strain. During batch cultures, important cell growth was observed for PPSg, PPSd and PPSc. In PPSg fermentation, the optical density increased from 0.2 to 2.5 after 60 h of growth when the *L. plantarum* had reached a stationary phase and was producing more acidity. The highest acidity was obtained from PPS supplemented by 20 g L^{-1} of glucose (PPSg fermentation).

The kinetic parameters were characterized by a maximal specific rate of growth ìmax of 0.136 h^{-1}, 0.105 h^{-1}, 0.107 h^{-1} and 0.07 h^{-1} respectively, for the growth of *L. plantarum* in PPS, PPSg, PPSd and PPSc. The strain seems to have a similar rate of growth for all fermentations. The maximal specific rate of sugar consumption Qsmax (Table 1) was much higher for culture in PPSd (4.57 $gg^{-1}h^{-1}$), followed by fermentation in PPSc (2.47), then in PPSg (2.26) and finally in PPS (1.55). The richness of date in fermentable sugar and growth factors promotes the growth of the strain. The maximal specific rate of lactic acid formation (QL.a max) evolves from 1.55 for PPS to 3.61 $gg^{-1}h^{-1}$ for PPSg (Table 1). The yield of conversion of sugar to biomass Yx/s was very high for culture in PPSd (0.1 gg^{-1}) compared to other

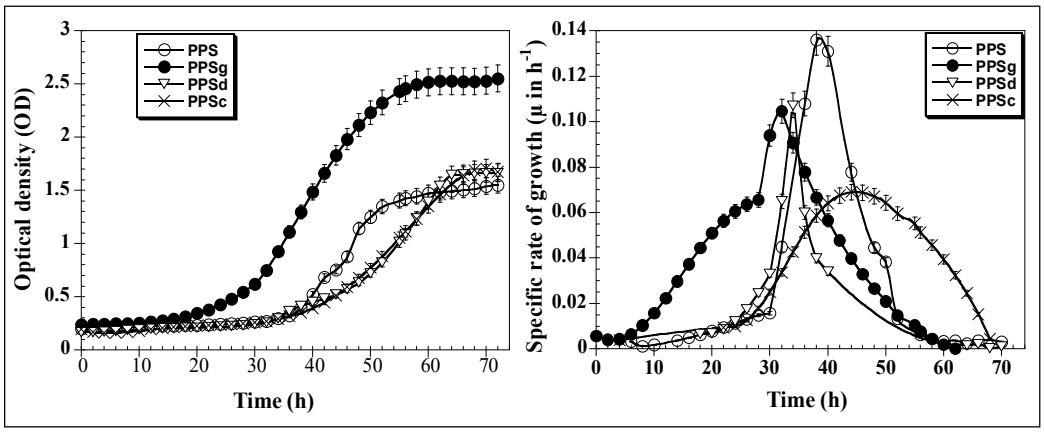

Fig 2: Kinetic profile of growth of *Lactobacillus plantarum* during batch fermentations. Data represent Mean±SD; n=3; Confidence level *p≤0.05*; PPS: Pod pea syrup; PPSg: PPS with 20 g of glucose; PPSd: PPS with 20 g of date; PPSc: PPS with 20g of carob pod.

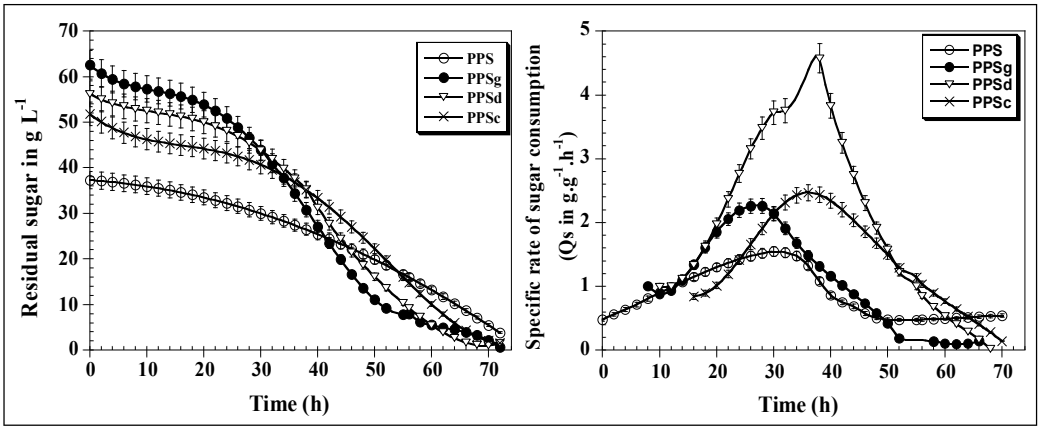

Fig 3: Kinetic profile of sugar consumption during batch fermentations. Data represent Mean±SD; n=3; Confidence level *p≤0.05*; PPS: Pod pea syrup; PPS g: PPS with 20 g of glucose; PPSd: PPS with 20 g of date; PPSc: PPS with 20g of carob pod.

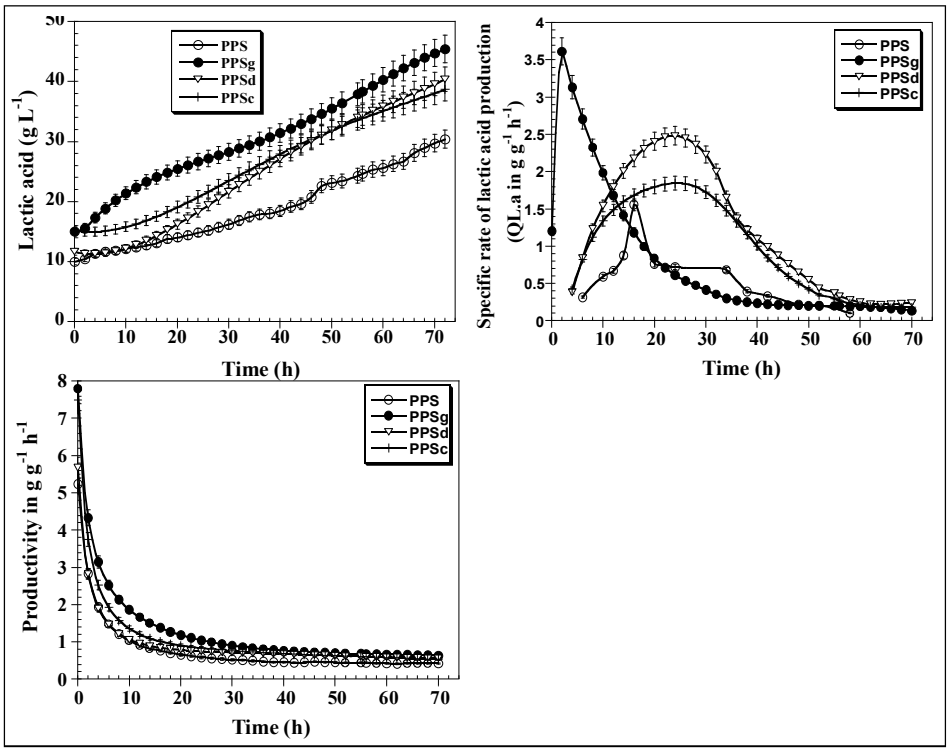

Fig 4: Kinetic profile of lactic acid production and productivity during batch fermentations. Values represent Mean±SD; n=3; Confidence level $p \leq 0.05$; PPS: Pod pea syrup; PPS g: PPS with 20 g of glucose; PPSd: PPS with 20 g of date; PPSc: PPS with 20 g of carob pod.

Table 1: Kinetic parameters of all batch fermentations.

Fermentations / Kinetic parameters	PPS	PPS g	PPSd	PPSc
Biomass formed	1.35±0.01	2.31±0.02	1.5±0.02	1.48±0.01
Sugar consumed (g L^{-1})	33.5±0.5	62±0.3	54.67±0.2	50.28±0.3
Lactic acid produced (g L^{-1})	20.4±0.2	30.4±0.2	28.76±0.1	23.5±0.2
µmax in h^{-1}	0.136±0.002	0.105±0.003	0.107±0.002	0.07±0.002
Qs max in g g^{-1} h^{-1}	1.55±0.001	2.26±0.002	4.57±0.001	2.47±0.001
QL.a max in g g^{-1} h^{-1}	1.55±0.001	3.61±0.001	2.48±0.002	1.85±0.001
Yx/s in g g^{-1}	0.02±0.002	0.05±.0.001	0.10±0.002	0.03±0.001
Yp/s in g g^{-1}	0.374±0.002	0.95±0.002	0.136±0.001	0.129±0.002
Productivity in g L^{-1} h^{-1}	5.24±0.1	7.79±0.2	5.67±0.3	7.5±0.3

Values represent Mean±SD; n=3; Confidence level $p \leq 0.05$; PPS: Pod pea syrup; PPS g: PPS with 20 g of glucose; PPSd: PPS with 20 g of date; PPSc: PPS with 20 g of carob pod.

fermentations. The higher yield of lactic acid YL.a/s and the higher value of lactic acid productivity were obtained from PPSg (Fig 4, Table 1).

CONCLUSION

In conclusion, we found that the essential oil displayed good antioxidant activity as determined by three methods due to the richness of this oil in different antioxidant substances. Further studies of chemical composition and antimicrobial activity of this potential oil are required in order to determine the active principle responsible for this strong antioxidant activity and to exploit it in the food and pharmaceutical industries. The pod pea was successfully used and valorized as medium for lactic acid production. The addition of glucose or other vegetable products rich in sugar, such as dates or carob, enhances the growth factors of fermentation.

Conflict of interest: None.

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