Journal of Chemistry

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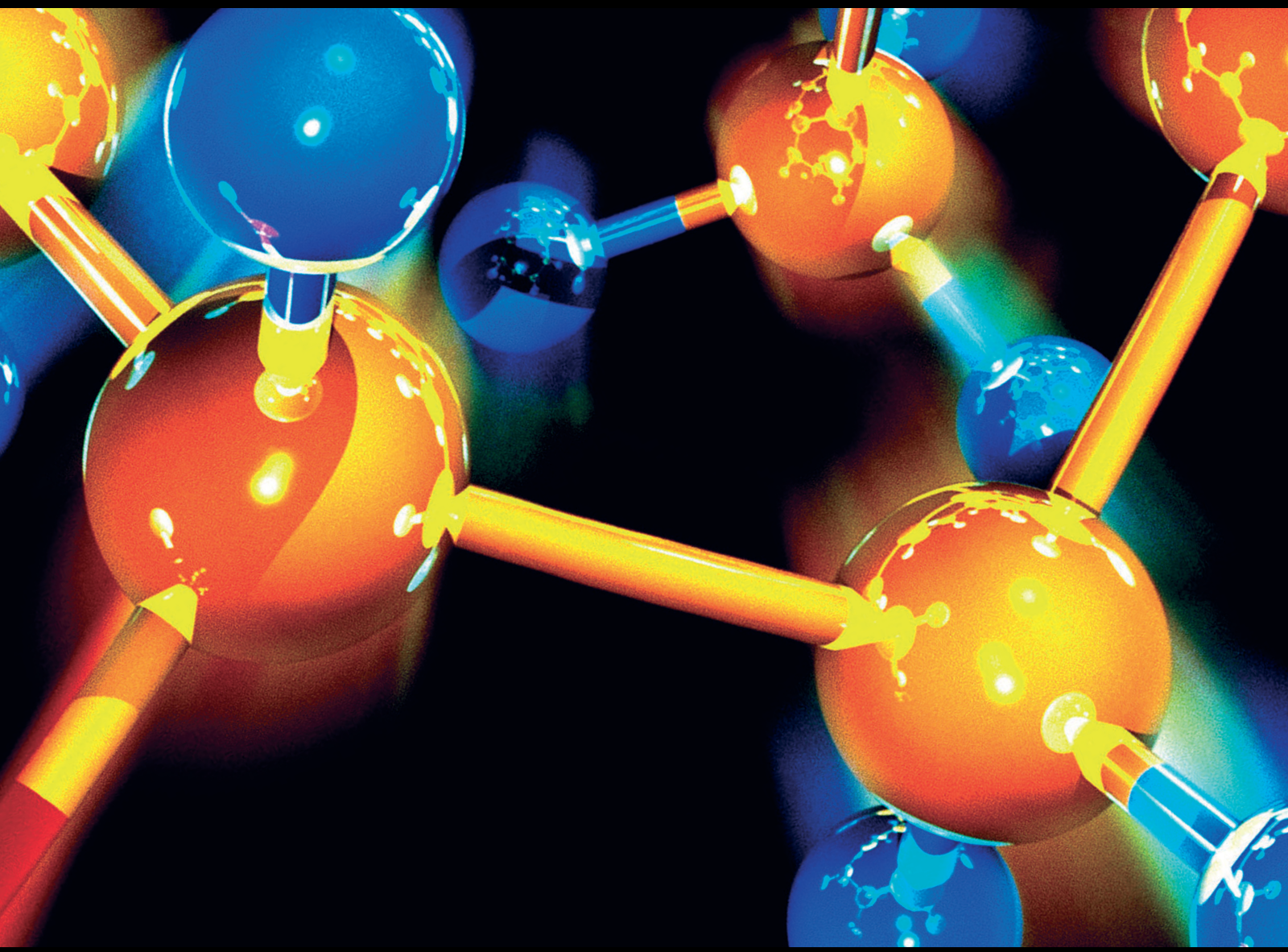

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Research Article

Plant Extract Valorization of *Melissa officinalis* L. for Agroindustrial Purposes through Their Biochemical Properties and Biological Activities

Mouna Souihi,[1,2] **Rayda Ben Ayed**,[3] **Imen Trabelsi**,[1] **Marwa Khammassi**,[4] **Nadia Ben Brahim**,[1] **and Mohamed Annabi**[1]

[1]*National Institute of Agronomical Research of Tunisia, Street of Heddy Karry 2049, Ariana, Tunisia*
[2]*Faculty of Sciences of Tunis, University Tunis El Manar, 2092 El Manar, Tunis, Tunisia*
[3]*Laboratory of Molecular and Cellular Screening Processes, Center of Biotechnology of Sfax, Address: B.P 1177, Sfax 3018, Tunisia*
[4]*Laboratory of Management and Valorization of Forest Resources, National Institute of Researches on Rural Engineering, Water and Forests (INRGREF), BP 10, Ariana 2080, Tunisia*

Correspondence should be addressed to Rayda Ben Ayed; raydabenayed@yahoo.fr

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Lemon balm (*Melissa officinalis* L.) is one of the rare medicinal plants in Tunisia. It was found only in two sites in the north of Tunisia with a small number of plants. The study of germination under the NaCl and PEG effect showed that Tunisian lemon balm seeds were sensitive to saline and osmotic stress. Morphological and biochemical characterizations of Tunisian *M. officinalis* were performed. Results showed that the Tunisian populations presented plants with long, broad leaves and weak branching. The major constituent in leaf essential oil was germacrene-D with a percentage ranging from 29.17 to 24.6%, and the major fatty acids were polyunsaturated fatty acids, linoleic acid, ranging from 73.93 to 66.74%. The phenolic content of *M. officinalis* extract varied significantly among origins which could explain the high variation in antiradical scavenging activity. The evaluation of allelopathic activities showed that the extract of the lemon balm leaves presented an allelopathic effect with the majority of the tested seeds.

1. Introduction

During the last two decades, research in herbal medicine has become one of the greatest scientific concerns [1]. In the socioeconomic context, the study of plants can lead to the achievement of adequate and low-cost therapeutic responses, combining proven scientific efficacy and cultural acceptability [2]. Located in the Mediterranean basin with great climatic variations from North to South, Tunisia presents a favorite terrain for the development of a flora rich in medicinal and aromatic species. More than 500 species out of a total of 2,200 (about 25% of the total flora) are considered for therapeutic use [3]. *Melissa officinalis* L. or lemon balm is a very rare medicinal species in a spontaneous area in Tunisia, classified among the 48 species identified as endemic rare and endangered according to the *IUCN* classification [4]. It has been encountered in few sites with a reduced number of plants in the forest region of Kroumirie and Mogods, having the geographical coordinates 8°–8°30′ East for the longitude and 36°15′–36° 45 ′North for the latitude [5]. Lemon balm is sought after for its lemony scent as well as for its many therapeutic activities. Since ancient times, it is used in cases of nervousness and minor sleep disorders, as well as in case of gastrointestinal disorders such as flatulence and abdominal pain, especially antidepressants [6]. In recent years, other uses have been studied, particularly in the field of plant protection products [7]. It is empirical that these properties have been attributed to it [8].

In Tunisia, there are no reports conducted on *M. officinalis*. Therefore, the aims of this study were to investigate, for the first time, the germination rate under abiotic stress and the morphological and chemical characterization and to

evaluate their antioxidant and allelopathic activities which may provide data for suitable conditions for cultivation of the best populations and for their agroindustrial exploitation.

2. Experimental

2.1. Plant Material. The plant material was botanically characterized by Dr Ben Brahim N. (Laboratory of Science and Agricultural Techniques, National Agricultural Research Institute of Tunisia (INRAT)) according to the morphological descriptions in the Tunisian flora. Several surveys were carried out before and after flowering (May and August) on the Tunisian territory according to the data of the geographical distribution of El Mokni et al. [9]. Tabarka and Nefza (Figure 1) cover almost the entire area of *M. officinalis* in Tunisia. Tunisian seeds were harvested from sites found in northern Tunisia. French seeds of *M. officinalis* were provided by the National Institute for Agricultural Research, France, and the German seeds were provided by the Institute for Food and Resource Economics (ILR), University of Bonn. The introduced seeds were used as references.

2.2. Study of Germination under Abiotic Stress. Seed germination is an essential process in the development of the plant. It is influenced by many abiotic factors such as salt and drought stress, which are perhaps the two most important abiotic constraints limiting plant development [10, 11]. In order to eliminate the germination inhibitors, the seeds, which had the same age, were sterilized in 0.5% sodium hypochlorite solution for 1 min and then washed with distilled water solution. Germination was performed in incubators with a photoperiod of 12 h (Sylvania white fluorescent lamps, 25 $mM{\cdot}m^{-2}{\cdot}s^{-1}$ photons with photosynthetically active radiation). Each treatment consisted of three replicates of 50 seeds. The seeds were germinated in NaCl solutions (0; 50; 100; 200; and 300 mM) and PEG 600 (polyethylene glycol) (0; −2.3; −4.6; −9.3; −13.9 bar) under the optimum temperature of 20°C [12]. The seeds were considered germinated during the appearance of radicals [13]. Sprouted seeds were counted and eliminated every two days over a 14-day period [14, 15].

2.3. Culture. The seeds of *M. officinalis* were grown on an experimental site at the National Institute of Agronomic Research of Tunisia (Ariana, 36°50′, 10°11′E), at 10 m altitude, with alkaline soil (pH = 8.7), and 450 mm of rain and upper semiarid bioclimatic stage. The experimental setup included lines spaced 1m and 50 cm apart between the plants. Each line comprised 10 individuals at the rate of three replicates for each origin. The average temperature was 25°C; the average monthly temperature varied between 15°C in January and 35°C in August. The plants were developed under biological conditions (no pesticide and fertilizer, weeds were eliminated manually, and additional irrigation if necessary was made). The cultivation was followed until the collection of the seeds.

2.4. Morphological Characterization. Eleven morphological characters were measured from 26 plants for each population. These characters relate to vegetative and reproductive developments. The choice of these characters was inspired by previous work done on several Lamiaceae plants such as *Lavandula* species [16].

2.5. Isolation of Essential Oil and GC/MS Analysis. The essential oils were obtained from 100 g (dry weight) of plant material using a Clevenger-type apparatus for 3 h. The hydrodistillation was performed in three replicates, and the oils were stored at 4°C until analysis by GC/MS. The average oil yields were estimated on the basis of the dry weight of the plant material. The oils were analyzed with a Hewlett-Packard 6890N/5975B inert GC-MSD system (Agilent, USA) equipped with two cap. columns, a HP-INNOWAX (30 m × 0.25 mm i.d., film thickness 0.25 μm), and a HP-5MS (30 m × 0.25 mm i.d., film thickness 0.25 μm) column (J&W Scientific, USA). The oven temperature was programmed isothermal at 50°C for 1 min, then increased from 50 to 250°C at 28/min, and finally held isothermal at 250°C for 15 min (injector temperature, 250°C; ion source temperature, 230°C; carrier gas, He (high purity 99.99%; 1.2 $ml{\cdot}min^{-1}$); injection volume, 1 μl; split ratio, 100 : 1). The electron impact ionization mode was used with an ionization voltage of 70 eV. Total ion chromatograms were obtained over the scan range of 30–800 a.m.u. in the full-scan acquisition mode, and the compounds were identified using the NIST05 and Wiley 7 databases with a resemblance percentage above 85%. Semiquantitative data were calculated from the GC peak areas without using correction factors and were expressed as relative percentage (peak area %) of the total volatile constituents identified. Retention indices (RIs) were determined for all the detected compounds based on the retention times (tr) of a homologous series of *n*-alkanes (C8-C30) [17, 18].

2.6. Fatty Acid Methyl Ester Preparation. Triplicate samples of 1 g of *M. officinalis* leaves were subjected to lipid extraction using a modified version of the Bligh and Dyer [19] method. Thus, leaf samples were kept in boiling water for 5 minutes and then ground manually using a mortar and pestle; chloroform/methanol mixture (2 : 1 v/v) was used for lipid extraction. After washing using fixation water, the organic layer containing lipids was recovered and dried under a nitrogen stream. Total fatty acids (TFAs) of total lipids were transmethylated using sodium methoxide solution (3% in methanol) [20]. Methyl heptadecanoate (C17 : 0) was used as an internal standard. The fatty acid methyl esters (FAMEs) obtained were subjected to GC analyses.

2.7. Phenolic Content and DPPH Radical Scavenging Assay. *M. officinalis* extracts were prepared with two solvents of ethanol and water. 20 g of plant powder was stirred in the presence of 200 ml of solvent for 72 h and then filtered. The filtrate was evaporated in the rotavapor [21]. The yield was calculated by the following formula:

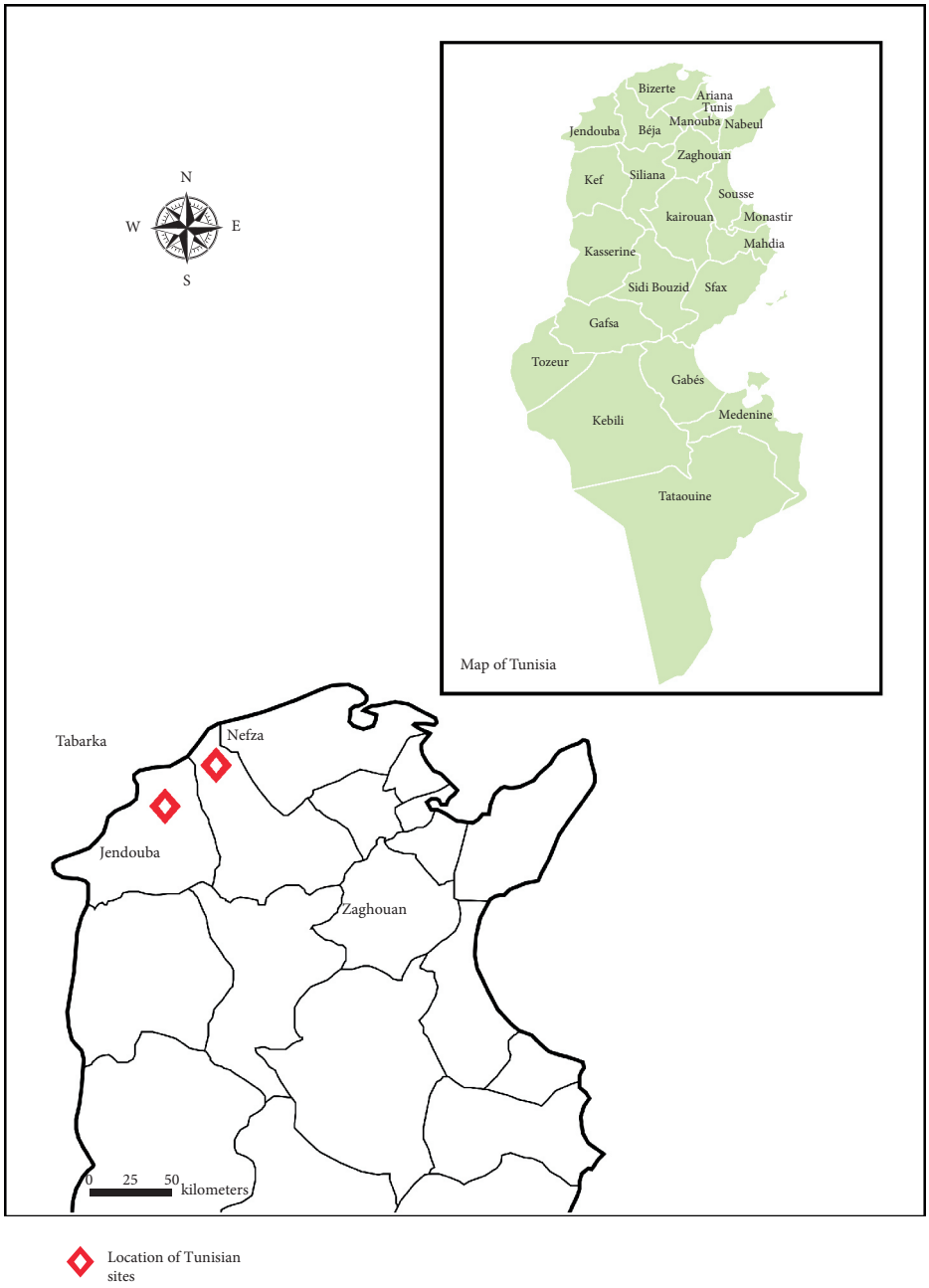

Figure 1: Location of *M. officinalis* sites in Tunisia.

$$\% \quad \text{yield} = \frac{\text{mass of the extract obtained}}{\text{mass of plant material before the extraction}} \times 100. \tag{1}$$

The total phenolic content of ethanolic and aqueous extracts was determined according to the method described by Lowman and Box and slightly modified by Moghaddam and Miran [22, 23]. About 100 μL of each sample was mixed with 46 mL of distilled water and 1 mL Folin–Ciocalteu reagent. The mixture was thoroughly shaken and left for 3 min before adding 2.9 mL of Na2CO3 (2%). After incubation for 2 hr in dark, the absorbance was measured at 760 nm. A standard curve of gallic acid was prepared. Total phenolic contents were expressed as milligrams gallic acid equivalents per gram of the essential oil (mg GAE/g DW) through the calibration curve with gallic acid. All samples were analyzed in triplicate.

The antioxidant activity was determined using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method according to Tohidi and Rahimmalek [24]. About 100 mL of each sample at different concentrations (between 50 and 800 μg/mL) was added to 2,500 mL DPPH ethanolic solution. The mixtures were shaken vigorously and then placed in the dark for 30 min. The absorbance of the solutions was measured at 517 nm. All samples were analyzed in triplicate. Butylated hydroxytoluene (BHT) was used as

positive control. The antiradical activity was expressed as IC_{50} (in mg/mL) which was defined as the concentration of the sample required to inhibit the formation of DPPH radicals by 50%. The percentage inhibition of the DPPH radical was calculated according to the following equation:

$$\%\,\text{inhibition} = \left[\left(\frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}}\right] \times 100, \quad (2)$$

where A_{control} is the absorbance of the DPPH solution without sample solution and A_{sample} is the absorbance of the sample after 30 min at 517 nm.

2.8. Allelopathic Activity. The extracts of lemon balm have been studied for their herbicidal effect. These extracts were tested on four seeds: radish (*Raphanus sativus* L.), fenugreek (*Trigonella foenum-graecum* L.), wild chicory (*Cichorium intybus* L.), medicago (*Medicago polymorpha* L.). The germination tests are carried out in an oven set at 25°C. For each test, four concentrations were used (0; 1; 3; and 5 mg/ml). The percentage of germination was observed daily for five days. Measurements of root length and shoot length were reported only at the end of the test (total germination of controls). The results represent the average of three replicates of 50 seeds for each treatment. To ensure their germination capacity, they were sterilized with 70% ethanol for 30 seconds, washed with sterile water, and then soaked in sodium hypochlorite for 20 minutes. Germination indices were determined by counting the number of seeds germinated at 24 hour intervals.

2.9. Statistical Analysis. All parameters were statistically described to determine means, standard deviations, and coefficients of variation by population. ANOVA (SAS version 9 software) was performed using the Duncan 5% test for each parameter. Principal component analysis (PCA) is one of the factor analysis methods that reduce data by defining the main axes (or principal components) from the monitored parameters. The observations are represented in relation to the main axes. Group analysis is performed by the ascending hierarchical classification (ACH) method. The graph representing the classification is a dendrogram of dissimilarity with standardized distances representing the nearest population in homogeneous groups. This analysis was performed using JMP®11.0 statistical software (SAS Institute, Inc., Cary, NC) with component analysis procedure.

3. Results and Discussion

3.1. Study of Germination under Abiotic Stress. The seed germination under different concentrations of sodium chloride showed that the germination rates of Tunisian origins and those introduced decrease when the sodium chloride concentration increases (Table 1). Exposure to osmotic conditions during the germination shows that Tunisian seeds are clearly sensitive to variations in water potential. The germination rate of Tunisian *M. officinalis* seeds decreases slightly at −2.3 bars and disappears from −4.6 bars. On the other hand, introduced seeds are relatively more tolerant (Table 2). Seeds from Germany have a germination rate above 40% at −4.6 bars. Introduced seeds, from Germany and France, show similar responses for the two treatments, tolerating the variation of NaCl and PEG than the Tunisian lemon balm seeds. We have little data about the tolerance of lemon balm seeds to abiotic stress. Germination of Tunisian seeds is studied for the first time in order to analyze the influence of salinity and osmotic potential on germination. Significant differences were observed between Tunisian and introduced seeds. Although the data were obtained from seeds sprouted in Petri dishes, the result may be related to in situ performance. Seeds from Tabarka and Nefza are more sensitive to salinity and to the osmotic potential, which explains the reduced number of *M. officinalis* plants in situ, where climatic constraints exert a natural selection pressure [25]. The germination rate of the lemon balm seeds studied is similar to the germination rate observed by Chartzoulakis and Klapaki, which demonstrated that 50 mM of salinity in the external environment delays germination in some plants but did not reduce the percentage of germination observed at the end of the experiment [26]. Similar results have been reported for *Atriplex griffithii* and *Cressa cretica* [27, 28]. Indeed, Iranian lemon balm seeds presented a germination rate that reaches 90% at 0 bar and 81% at −2 bar [29]. The reduction of germination rate under salt stress and their toxic and osmotic effects involved in germination have been demonstrated by several authors [25, 30]. In the case of Tunisian lemon balm seeds based on the results obtained, it is suggested that the osmotic effect of NaCl is responsible of the disturbance of seed germination. So, the osmotic effect, which leads to reduced water absorption, may be responsible for the inhibition of germination seeds from Tunisia.

3.2. Morphological Characterization. The morphological characterization of *M. officinalis* from the four origins, Tabarka, Nefza, Germany, and France, was addressed by statistical analysis carried out on 11 agro-morphological characters measured on plants cultivated under homogeneous conditions. One-way analysis of variance (population effect) showed significant differences for most of the traits studied (Table 3). To understand the variance sources of *M. officinalis*, a principal component analysis (PCA) was performed by grouping together the seven significant morphological characters in the first two axes describing 79.3% of the total variation (Figure 2(a)). The plot drawn along the two axes showed three distinct groups of plants (Figure 2(b)). The first group consisted of the plants of the German population, which was characterized by plants with good vegetative development presenting the tallest plants (43.34 cm), the most branched, and the highest number of leaves and flowers. The Tunisian populations (Tabarka and Nefza) formed the second group, which was characterized by plants with long, broad leaves and weak branching. The third group was formed by the plants from France population, which was distinguished by a weak vegetative development. Hierarchical analysis based on morphological characters

Table 1: Effect of NaCl on seed germination of *M. officinalis*.

NaCl (mM)	0	50	100	200	300
Tabarka	98.33a	85b	0b	0	0
Nefza	100a	88.33b	10b	0	0
France	100a	93.33b	48.33a	0	0
Germany	100a	100a	58.33a	0	0

Means in each column followed by the same letters are not significantly different ($P > 0.05$).

Table 2: Effect of PEG on the germination rate of *M.officinalis* seeds.

Bars	0	−2.3	−4.6	−9.3	−13.9
Tabarka	100a	91.66a	0c	0	0
Nefza	100a	90a	0c	0	0
France	100a	86.66b	13.33b	0	0
Germany	100a	63.33c	41.66a	0	0

Means in each column followed by the same letters are not significantly different ($P > 0.05$).

using the Euclidean square method allowed establishment of the relationship between lemon balm of different origins. The morphological study showed a large phenotypic diversity between the populations for the majority of the agromorphological characters. This indicated the existence of a wide range of genetic variability for the traits studied and highlighted the potential from genetic improvement using such a gene pool. Sari and Ceylan also observed a great variability of morphological characters in 11 populations of *M. officinalis* from different regions of Turkey and European countries [31]. This may be due to cross pollination of lemon balm.

3.3. Essential Oil. The *M. officinalis* samples cultivated under the climatic conditions of the INRAT yielded a small amount of essential oil. The oils were analyzed by GC/MS. The chemical composition of the four *M. officinalis* from different origins was reported in Table 4. Chemical analysis of the essential oil samples conducted according to their retention time (RT) revealed the presence of 42 compounds, representing about (86.11%, 83.1%, 96.72%, and 71.88%) of the total oils obtained from Nefza, Tabarka, Germany, and France, respectively. In addition to the differences in the essential oil yield, the GC/MS analyses revealed qualitative and quantitative differences in the composition between the oils of the four origins. GC/MS analysis showed that the oils of the German population were characterized by the presence of 39.31% of geranial and 27.71% neral, which were the dominant components, and 12.23% of β-caryophyllene. The sesquiterpene caryophyllene oxide (27.06%) was found to have the highest value in the French population, which exhibited lower levels (7.12–4.29%, respectively) of geranial and neral. Germacrene-D (32.08–27.06%) was the highest in the Tunisian samples from Tabarka and Nefza, together with the sesquiterpene caryophyllene (16.4–14.7%, respectively). Considering the fact that minor components of the essential oils were less important for a comparison, only the major components representing more than 2% of the essential oil composition were taken into account to illustrate the variation of the components content from different origins [17].

In order to investigate the similarity and relationship between essential oil compositions of our studied samples, a hierarchical cluster analysis (HCA) was performed based on the components. The dendrogram of HCA classified the oils of the Tunisian populations of Tabarka and Nefza in the same group. This group was characterized by the predominance of germacrene-D as the major compound and emphasized the distinctiveness of the French oil, rich in caryophyllene oxide. The third group was formed by the German oil characterized by citral (neral and geranial) (Figure 3).

3.4. Fatty Acids. To the best of our knowledge, the foliar fatty acid composition of *M. officinalis* was reported herein for the first time. The proportion of the majority of fatty acids did not show differences according to the origin of samples (Table 5). *Melissa officinalis* leaves were characterized by a high proportion of polyunsaturated fatty acid (PUFA), linoleic acid (73.93–66.74%), versus (16.25–13.32%) saturated ones (SFA), oleic acid, and (6.29–4.26%) of monounsaturated (MUFA) palmitic acid. The comparison between different lemon balm evidenced a similarity, at least with reference to the presence of the main fatty acid constituents. It is noteworthy that previous findings showed that the genera *Satureja, Origanum*, and *Thymus* of the Lamiaceae family had some minor variations in fatty acid composition, which were dominated by the chemotypes of linoleic acid, palmitic acid, and linolenic acid [32].

3.5. Total Phenolic Content and Antioxidant Activity. Total phenolic contents in lemon balm leaves from different origins are shown in Table 6. The amounts of total phenolics in *M. officinalis* varied significantly among populations. Ethanolic extract from Tunisian populations Tabarka and Nefza showed the highest amount of phenolic compounds (0.94 mg GAE/g DR and 0.87 mg GAE/g DR, respectively). However, the lowest content was observed in the aqueous extract from German population (0.2 mg GAE/g DR) (Figure 4).

The extraction yield was influenced by the polarity of the solvent; according to Bourgou, the water (5.2) is more polar than ethanol (9.0), which is why the yield ethanol extraction is lower than the water [33]. When comparing values in this study to the literature, there was considerable variation in total phenolic contents reported of lemon balm from different origins. Boneza and Niemeyer indicated that the average total phenolic contents for the five lemon balm cultivars from the USA ranged from 5.50 ± 1.03 mg GAE/g DW to 26.87 ± 1.93 mg GAE/g DW [34]. Wojdyło et al. and Dastmalchi and Dorman reported that unspecific lemon balm showed total phenolic contents from 0.13 to 269 mg GAE/g DW [35, 36]. Boneza and Niemeyer noted much higher total phenolic contents for the lemon balm cultivars, with values ranging from 359 mg GAE/g DW for the "Lorelei" cultivar to 427 mg GAE/g DW for "Gold Leaf" [34]. This variability in lemon balm total phenolic contents across studies was therefore most likely due to differences in plant

Table 3: Analysis of variance in *Melissa officinalis* populations based on agromorphological traits.

	Mean of square (MS)										
Source of variation	Plant height (PH)	No. of branches/ plant (nB)	Stem diameter (SD)	Leaf length (LL)	No. of leaves (nL)	Leaf width (LW)	No. of nodes (nN)	No. of flowers (nF)	Fresh weight (FW)	Dry weight (DW)	Seed yield/ plant (SY)
Populations	511.36974**	295.07169***	1.05966***	8.09158***	6327.01563*	3.24630***	1.58592ns	12655.95356***	70.89806 ns	7.56814 ns	0.51593 ns

*, **, and *** denote statistical significance at 0.05, 0.01, and 0.1 levels, respectively. ns: not significant.

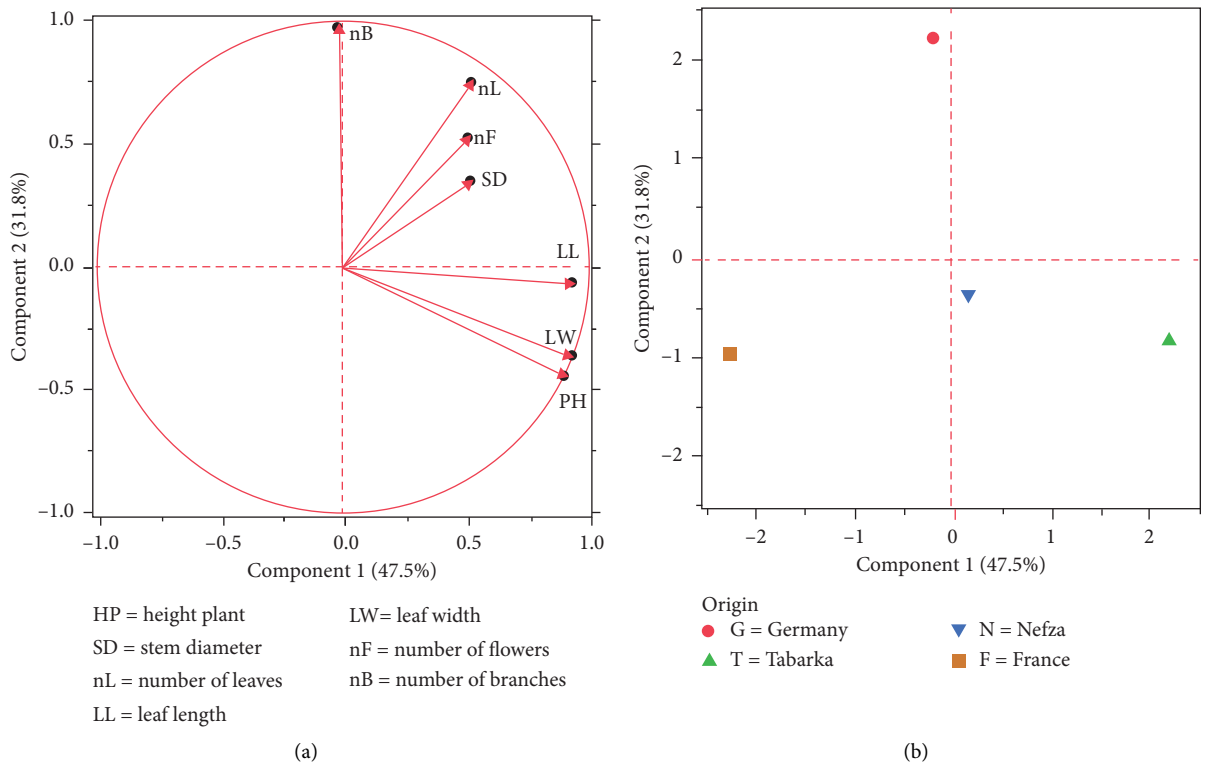

Figure 2: Plot of the first two principal components (C1 and C2) for the PCA of *M. officinalis*.

Table 4: Comparison of the essential oils isolated from different origins of *M. officinalis*.

No	Components	RI	Content (%)			
			Nefza	Tabarka	Germany	France
1	Camphene	954	—	—	—	1.29
2	£-3-Carene	1011	0.32	—	—	—
3	(Z)-α-Ocimene	1026	—	0.5	—	—
4	Citronellol	1229	—	—	1.88	—
5	Neral	1240	—	—	27.71	4.29
6	Geranial	1267	—	—	39.31	7.12
7	Thymol	1290	—	—	0.4	—
8	α-Ylangene	1372	0.42	—	—	—
9	α-Copaene	1376	0.72	0.54	—	—
10	Geranyl acetate	1381	—	—	1.42	—
11	Dehydro-ar-ionene	1389	0.84	—	—	—
12	(E)-α-Bergamotene	1412	0.55	—	—	1.24
13	(E)-Caryophyllene	1419	1.36	1.25	—	1.06
14	B-Caryophyllene	1420	14.7	16.4	12.23	8.92
15	α-Cedrene	1432	0.52	0.27	—	—
16	Alloaromadendrene	1439	—	0.59	—	—
18	Aromadendrene	1441	0.3	—	—	—
19	α-Cubebene	1475	1.45	1.34	1.23	0.42
20	Germacrene D	1468	27.06	32.08	1.67	2.0
21	Bicyclogermacrene	1495	0.18	—	—	—
22	cis-Calamenene	1521	0.75	—	—	—
23	B-Sesquiphellandrene	1522	2.75	0.4	—	0.97
24	delta-Cadinene	1523	0.73	—	—	—
25	α-Cadinene	1524	0.34	—	—	—
26	gamma-Cadinene	1538	4.96	—	0.76	1.77
27	α-Calacorene	1542	0.71	1.23	—	0.76
28	Nerolidol	1559	0.9	—	—	—

Table 4: Continued.

No	Components	RI	Content (%)			
			Nefza	Tabarka	Germany	France
29	Globulol	1568	0.42	—	—	—
30	Caryophyllenol	1572	0.91	1.47	0.5	2.23
31	Germacrene D-4-ol	1574	0.72	—	—	—
32	Caryophyllene oxide	1576	9.54	16.61	8.76	27.06
33	Spathulenol	1578	0.49	—	—	—
34	Humulene oxide II	1606	0.56	1.01	0.26	1.29
35	α-Cadinol	1654	4.61	3.24	—	5.64
36	t-Muurolol	1627	—	—	0.59	—
37	Isoaromadendren epoxide	1641	0.77	—	—	0.46
38	Farnesol	1743	—	0.49	—	—
39	(β-Z) Curcumen-12-ol	1756	0.53	—	—	—
40	Phytol	1949	6.96	5.68	—	3.64
41	Epi manoyl oxide	1993	0.22	—	—	—
42	(E-E)-geranyl linalool	2027	0.82	—	—	1.59
	Total compound (%)		86.11	83.1	96.72	71.88

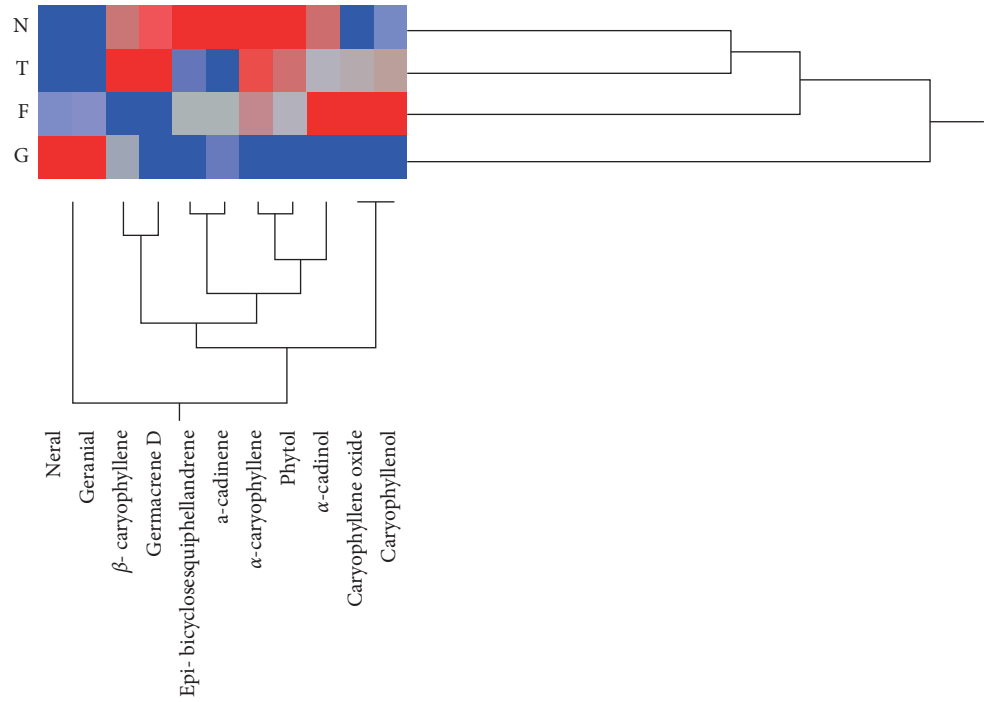

Figure 3: Total phenolic content according to the origin of *M. officinalis*. N: Nefza, T: Tabarka, F: France, and G: Germany.

Table 5: Fatty acid percentage content of *M. officinalis* leaves.

Fatty acid	Tabarka	Nefza	France	Germany
C16: 0 (palmitic acid)	15.82	13.32	16.25	15.77
C16: 1 (palmitoleic acid)	1.00	1.47	—	1.71
C18: 1$n-9$ (oleic acid)	5.89	4.26	4.62	6.29
C18: 2$n-6$ (linoleic acid)	70.75	66.74	73.93	74.08
C20: 0 (arachidic acid)	1.19	1.31	1.60	1.06

Table 6: Yield of aqueous and ethanolic extracts of *Melissa officinalis*.

Origin	Aqueous extract (%)	Ethanolic extract (%)
Tabarka	31a	6b
Nefza	29.05a	7b
France	28.5a	7.5b
Germany	26.5a	7.5b

Means in each column followed by the same letters are not significantly different ($P > 0.05$).

materials and extraction conditions. Few studies within the literature have evaluated the effect of seed source on plant phenolic levels. Antioxidant capacities of lemon balm were determined using the DPPH assays, and results are shown in Table 7. There was significant variation in free radical scavenging activities among populations, and the inhibitory concentrations IC50 ranged from 123,29 to 541,81 mg/mL

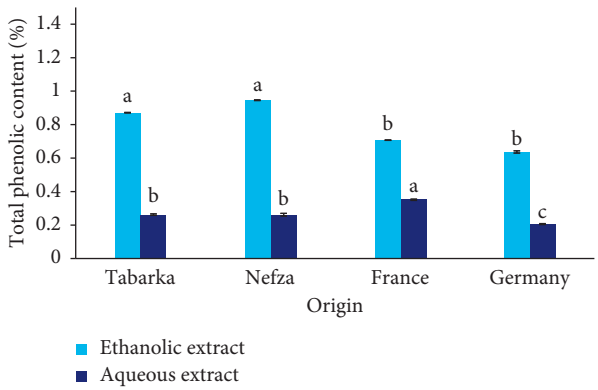

Figure 4: IC_{50} of the ethanolic extract according to the origin of *M. officinalis* and seeds. Values followed by the same letters are not significantly different ($P > 0.05$).

Table 7: DPPH radical scavenging capacity (IC_{50}) of ethanolic and aqueous extract.

Origin	IC_{50} DPPH (mg/mL)	
	Ethanolic extract	Aqueous extract
Tabarka	$123.29^{d} \pm 0.22$	$514.69^{b} \pm 0.20$
Nefza	$175.64^{c} \pm 0.16$	$496.54^{c} \pm 0.45$
France	$350.85^{b} \pm 0.16$	$340.28^{d} \pm 0.27$
Germany	$541.81^{a} \pm 0.33$	$645.57^{a} \pm 0.32$

Means in each column followed by the same letters are not significantly different ($P > 0.05$).

Table 8: IC_{50} of the aqueous extract according to the origin of *M. officinalis* and seeds tested.

IC_{50}	Medicago	Wild chicory	Radish	Fenugreek
Tabarka	3.82^{a}	5.67^{c}	2.92^{c}	21.32^{a}
Nefza	3.63^{a}	5.34^{c}	2.79^{c}	18.33^{a}
France	2.66^{b}	6.55^{b}	6.05^{b}	5.71^{c}
Germany	2.59^{b}	7.85^{a}	9.77^{a}	16.26^{b}

Means in each column followed by the same letters are not significantly different ($P > 0.05$).

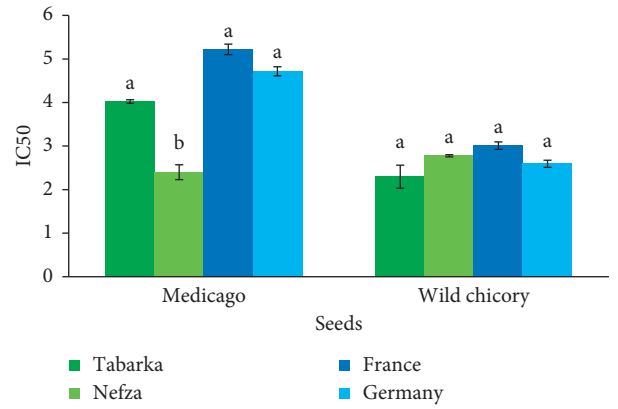

Figure 5: IC_{50} of the ethanolic extract according to the origin of *M. officinalis* and seeds. Values followed by the same letters are not significantly different ($P > 0.05$).

for the ethanolic extract and from 340,28 to 645,57 for the aqueous extract.

In accordance with the data obtained from this study, Aissi et al. reported the effect of geographic origin on antioxidant activity of essential oils [37]. Indeed, these variations were probably due to differences in phenolic compound content [38, 39].

In fact, phenolics, due to their hydroxyl groups that allow them to donate hydrogen to DPPH free radicals, were considered as the major factor contributing to antioxidant activity of plants [40]. Several studies reported by Hammoudi et al., Habellah et al., and Tlili et al. showed a correlation between total phenolic content and antioxidant activity [41–43]. Cultivar also influenced DPPH antioxidant capacity ($P = 0.002$) with "Lemonella" ($45.2 \pm 11.2\ \mu$mol TEAC/g DW) having significantly lower DPPH free radical scavenging than all other cultivars in the study. "Lime" balm also had the highest DPPH antioxidant capacity, $189.5 \pm 19.7\ \mu$mol TEAC/g DW. Samples having higher phenolic levels typically exhibit greater antioxidant capacity [44].

3.6. Allelopathic Activity. The inhibitory effect of the aqueous extract of *Melissa officinalis* from different origins on seed germination was clearly visible from the lower concentrations. In the case of Medicago, the aqueous extract of lemon balm had the highest inhibition levels (Table 8). The ethanol extract was tested only on the seeds of medicago and chicory, given the low yield of extract. The results showed that the rate of germination was strictly concentration dependent (Figure 5). The study of IC_{50} of ethanolic extract from the four origins showed close concentrations with chicory seeds. However, with the medicago seeds, the ethanol extract from Tunisian *M. officinalis* showed the lowest IC_{50}. To follow the effect of the ethanolic extract of Tunisian Lemon balm on the development of seedlings, the length of the root and the shoot were measured under the effect of different concentrations. The longest root was recorded in untreated seeds, and the shortest length was recorded in seeds treated with 5 mg/ml. For the shoot, the ethanolic extract of lemon balm significantly reduced its length depending on the concentration in both species. Variance analysis showed significant differences ($P < 0.05$) between treatments (Tables 9 and 10). From a physiological point of view, germination begins with the imbibitions of the seed and ends with the beginning of growth marked by the lengthening of the root [45]. The germination of a seed can take place only if certain favorable conditions are met: oxygen, temperature, and water. Moreover, it is well known that natural substances produced by plants are able to delay or inhibit seed germination and seedling growth. This explains the effects of aqueous and ethanolic extracts of *M. officinalis* on the germination of certain seeds. The results obtained from the germination tests showed the existence of an allelopathic phenomenon under experimental conditions. This provided evidence that some plants contain chemical compounds with herbicidal activity. The aqueous extract of lemon balm from Tunisia was shown as the most inhibitor against the majority of seeds tested. The ethanolic extract showed a greater allelopathic power against the Medicago seeds than on the chicory seeds. The allelopathic effect may depend on the species of seeds tested, which was provided by

Table 9: Effect of ethanol extract on the germination of medicago seeds.

	Nefza			Tabarka		
	Inhibition rate (%)	Shoot	Root	Inhibition rate (%)	Shoot	Root
0 mg/ml	0	3.57a	3.25a	0	3.57a	2.62a
1 mg/ml	25	2.45b	2.55b	10	2.37b	2.80a
3 mg/ml	31.66	1.97c	2.52b	30	1.47c	2.05a
5 mg/ml	38.33	1.47d	1.77c	78.33	0.92c	1.20b
IC50	4.06			2.3		

Means in each line followed by the same letters are not significantly different ($P > 0.05$).

Table 10: Effect of ethanol extract on the germination of wild chicory seeds.

	Nefza			Tabarka		
	Inhibition rate (%)	Shoot	Root	Inhibition rate (%)	Shoot	Root
0 mg/ml	0	2.02a	3.25a	0	1.90a	3.35a
1 mg/ml	56.66	0.70b	1.07b	60	0.95b	0.70b
3 mg/ml	61.66	0.25c	0.45c	73.33	0.67b	0.55b
5 mg/ml	68.33	0.22c	0.30c	76.66	0.20c	0.25b
IC50	2.39			2.65		

Means in each line followed by the same letters are not significantly different ($P > 0.05$).

Serghini et al. [46], where sunflower extract (*Helianthus annus* L) had an effect on the germination of *Orobanche ramose (Phelipanche ramose* L.) but had no effect on the germination of *Orobanche cernua* Wallr. The results obtained corroborated with those found by Kato-Noguchi and Ino in which certain fractions of a hydroacetonic extract of *M. officinalis* inhibited the germination and the growth of roots and shoots of A*maranthus caudatus* L., *Lepidium sativum* L., *Digitaria sanguinalis* L., *Phleum pratense* L, *Lactuca sativa* L., and *Lolium multiflorum* Lam. [6]. The aqueous extract is the most effective with an IC_{50} being 0.14 mg/ml.

4. Conclusion

Exploration of native plant genetic resources is one of the main objectives of the Medicinal and Aromatic Plants Program. It also aims to evaluate and conserve rare spontaneous plants, genetic resources, and especially threatened species in their natural environment. The seeds of Tunisian lemon balm were the most sensitive to salinity and the variation of the osmotic pressure, which explains the reduced number of plants in situ. The morphological study showed that Tunisian lemon balm was characterized by small plants with a weak branching. The aqueous and ethanolic extracts of this species had an interesting allelopathic activity, which allowed the use of lemon balm in the field of phytosanitary products as weed killers.

Data Availability

All data generated or analyzed during this study are included in this published article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Supplementary Materials

Supplement 1: morphological characters; Supplement 2: raw data of morphological studies; Supplement 3: dendrogram established from Euclidean squared for standard variables using the average method based on agromorphological traits among four populations of *M. officinalis*. (*Supplementary Materials*)

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Research Article

Bioreactor Scale-Up and Kinetic Modeling of Lactic Acid and Biomass Production by *Enterococcus faecalis* SLT13 during Batch Culture on Hydrolyzed Cheese Whey

Manel Ziadi,[1] **Sana M'Hir**,[1] **Abdelkarim Aydi**,[2] **and Moktar Hamdi**[1]

[1]*Laboratory of Microbial Ecology and Technology (LETMi), The National Institute of Applied Sciences and Technology (INSAT), Carthage University, 2 Boulevard de la Terre, BP 676, 1080, Tunis, Tunisia*
[2]*Laboratory of Materials Molecules and Applications, Preparatory Institute for Scientific and Technical Studies, Carthage University, BP 51, La Marsa, 2075, Tunis, Tunisia*

Correspondence should be addressed to Manel Ziadi; manel_z52@yahoo.fr and Sana M'Hir; sana2617@yahoo.fr

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Kinetic modeling of biomass and lactic acid production by *Enterococcus faecalis* SLT13 have been developed during batch culture in M17 and Hydrolyzed Cheese Whey (HCW) in 2 L and 20 L bioreactors. The specific growth rate μ_{max} was higher in 20 L bioreactor (1.09 h^{-1}); however, the maximum specific lactic acid production rate $q_p$max and maximum specific sugar utilization rate $q_s$max were higher in 2 L bioreactor. Biomass and sugar utilization were affected by lactic acid inhibition in HCW. No effects of substrate inhibition have been observed. Substrate limitation of biomass has been observed on HCW in 20 L bioreactor; the substrate limitation constant for biomass K_{sx} was 4.229 g/L. Substrate limitation of sugar consumption has been observed on M17 in 2 L bioreactor; the substrate limitation constant for sugar consumption K_{ss} was 2.73 g/L. Compared to experimental data, the model provided good predictions for biomass, sugar consumption, and lactic acid production.

1. Introduction

Actually, the lactic acid and its derivative polylactic acid (PLA) market appears among the most important markets of the chemical industry. This market is in clear progress and it is predictable to attain USD 3.82 billion and 5.16 billion on 2020 for the lactic acid and the PLA, respectively. This growth is due to several reasons: the increasing request for the eco-friendly products, the increasing prices of products derived from the petrochemistry, and the limited reserves of fossil energy [1].

The lactic acid presents several applications, essentially the food industries (preservative and acidulant), textile, and pharmaceutical industries. Moreover, the expansion of PLA market is the main reason for lactic acid market rising. Indeed, the lactic acid is used as monomer for PLA production [2]. For its bioavailability and biodegradability, the PLA is used not only in the food industries for packaging of sensitive foods (i.e., eggs) and tablets in the pharmaceutical industry, but also in electronics and textile, which can be substituted for synthetic polymers derived from petrochemistry.

Two processes are used to produce industrially lactic acid: chemical synthesis and microbial fermentation. However, microbial fermentation presents numerous advantages compared to chemical synthesis; these advantages are essentially environmental; fermentation is a clean process; also the lactic acid obtained by fermentation is optically pure (L (+)-lactic acid) compared to the racemic mixture (50% L-lactic acid and 50% D-lactic acid) obtained chemically [3]. Indeed, PLA with L (+)-lactic acid content higher than 90% is a crystalline polymer and is preferred for commercial uses than the amorphous polymer with high content of the D-isomer [4].

For advantageous commercial process, choosing an appropriate carbon source for lactic acid production is crucial. Indeed, a promising substrate must be of low cost, does not contain contaminants, and allows fast growth of the lactic acid producer microorganism as well as high

recovery in lactic acid [5]. Thus, several substrates have been used for the production of lactic acid through microbial fermentation. Among them, cheese whey, a major biowaste of the dairy industry, is a good candidate for this purpose. This byproduct contains 5–8% (w/w) of dry matter, in which lactose is approximately 60–80%, proteins 10–20%, minerals, vitamins, fat, lactic acid, and trace elements [6]. Cheese whey has been widely used for various productions including organic acids, single-cell proteins, enzymes, and ethanol [7].

Many microorganisms are involved in lactic acid production from varied feedstock including Lactic Acid Bacteria (LAB), yeasts, and fungi. LAB species used for lactic acid production include *Lactobacillus*, *Streptococcus*, and *Enterococcus* producing L(+)-lactic acid, while *Leuconostoc* and *Lactobacillus bulgaricus* produce D(−)-lactic acid [8]. Genetically modified *Saccharomyces cerevisiae* was used for the production of lactic acid in continuous fermentation [9]. *Rhizopus* is the major fungi used for lactic acid production; the species *R. oryzae* and *R. microsporus* have been previously used and high amounts of lactic acid were formed [10]. Others bacterial strains, *Bacillus* and *Escherichia coli*, were also used for the production of both lactate isomers [11, 12].

Among lactic acid bacteria involved in lactic acid production, *Enterococcus faecalis* has been described in many studies as lactic acid producer. *Enterococcus faecalis* RKY1 is able to produce lactic acid from agricultural feedstock, such as wheat, barley, corn, hydrol, soybean curd residues, malt, and from single and mixed sugars [13–15]. The same strain was used by Nandsana and Kumar [16] to produce lactic acid from molasses. *Enterococcus faecium* No. 78 has been used for lactic acid production from liquefied sago starch [17]. *Enterococcus faecalis* QU11 is able to produce lactic acid from glycerol fermentation [18].

With increasing interest in the industrial application of lactic acid and PLA, different kinetic models have been developed in order to study the growth and lactic acid production by different species of LAB. Indeed, kinetic modeling is an essential step to optimize a fermentation process, as though models help in process control, reducing process costs and time, and improving product quality [19].

Earlier studies have described lactic acid production by *Lactobacillus* sp. using Luedeking–Piret model. This kind of model relates cell growth and lactic acid production by including two coefficients: growth-associated coefficient (α) and a non-growth-associated coefficient (β). Unstructured kinetic models such as the Gompertz equation have been also used [20]. Further developments have proposed models which included Monod equation to describe the relation between a limiting substrate and biomass growth and then product formation [21, 22]. Monod equation relates the specific growth rate (μ) to the concentration of a single growth-limiting substrate (S) via two parameters, the maximum specific growth rate (μ_{max}) and the substrate affinity constant (K_S). Other authors have proposed the inhibitory effects of high initial lactose concentrations, lactose limitation, and lactate inhibition [23, 24], while others developed models showing the inhibition effects of both associated and dissociated forms of lactic acid [25, 26].

This study is conducted to find the most suitable model that describes biomass and lactic acid production by *Enterococcus faecalis* SLT13 in batch cultures in a medium prepared with hydrolyzed cheese whey and in a synthetic medium (M17). The effects of culture media and scale-up from 2 to 20 L bioreactors on the kinetic parameters were studied.

2. Materials and Methods

2.1. Bacterial Strain and Culture Conditions. The strain *Enterococcus faecalis* SLT13 used in this work was isolated from Tunisian traditional fermented milk "Leben" [27]. Stock cultures of this strain were stored in M17 broth containing 20% glycerol (Merck) at −80°C.

Enterococcus faecalis cells were grown in M17 broth (Merck), containing glucose as main carbon source or in papaïn Hydrolyzed Cheese Whey (HCW). Cheese whey powder (BHA, Belgium SA) was rehydrated in distilled water (6% w/v). All fermentations were supplemented with (/L): 2 g yeast extract (YE); 0.5 g NH_4Cl; 2 g K_2HPO_4; and 25 mg $MnSO_4$. Papaïn was provided by the Walloon Center of Industrial Biology (CWBI, Belgium) and had an activity of 22 000 IU. The enzyme : substrate ratio was 0.5 : 100 (w/w). The whey hydrolysis was carried out at pH 5.0 and 60°C. The enzyme was inactivated by thermal treatment (90°C for 5 min) and cooled in an ice bath. Hydrolysis time was 30 min. After hydrolysis, the pH was adjusted to 7.0.

Batch cultures using M17 or HCW media were carried out in 2 L bioreactor Biostat B (B. Braun Biotech International, Melsungen, Germany). A working volume of 1.5 L was used, which comprised 1.4 L growth medium and 100 mL of inoculum. Inoculum was prepared by adding 100 μL of the preserved strain in 250 mL Erlenmeyer flask containing 100 mL of M17 broth (approx. 1×10^7 CFU/mL) and then incubated at 30°C. The media used were sterilized in the bioreactor at 121°C for 15 min. The set point of pH was 7.0 ± 0.2, temperature 30°C, and stirrer speed 100 rpm. The pH was adjusted with 1 N HCl or 1 N NaOH. The fermentation was run for 10 h in M17 and 24 h in HCW. Samples were withdrawn every 2–4 h for determination of glucose, lactose, and lactic acid concentrations and viable cells count (CFU/mL).

Batch culture in 20 L bioreactor was conducted as follows: 100 μL of the preserved strain was inoculated into 20 mL vial containing 10 mL M17 broth and then incubated at 30°C for 12 h and transferred again in 250 mL Erlenmeyer flask containing 200 mL M17 broth (preculture P_1) that was incubated at 30°C for 16 h. The preculture P_1 from the exponential growth phase was inoculated into 1 L Erlenmeyer flask containing 800 mL M17 broth (preculture P_2). Batch culture was performed in 20 L stirred bioreactor (Biolafitte, France) in HCW. The media used were sterilized in the bioreactor at 121°C for 15 min. The preculture P_2 in the exponential growth phase prepared in 1 L Erlenmeyer flask was added to the culture medium. The temperature of the bioreactor was maintained at 30°C and the pH was controlled at 7 ± 0.2 by the addition of NaOH (3N). Samples were withdrawn every 2–4 h for determination of lactose and lactic acid concentrations and viable cells count (CFU/mL). All experiments were conducted in triplicate.

2.2. Biomass Determination. For biomass determination, a calibration curve relating dry cell weight with the cell concentration (estimated by viable cells counting method) was used. For cells counting, 0.1 mL of diluted sample was spread over M17-agar surface. The plates were then incubated for one night at 30°C. The number of colonies was counted and expressed in CFU/mL (Colony-Forming Units/mL).

2.3. Analytical Analysis. Glucose, lactose, and lactic acid concentrations in the fermentation broth were measured by HPLC (Agilent Technologies) using an ion-exclusion column (SupelCo Gel C-610H) operated at 35°C. Components were eluted with 0.1% phosphoric acid at a flow rate of 0.5 mL·min^{-1}. Detection was accomplished by a refractive index detector.

2.4. Kinetic Model

2.4.1. Model Development. The model used in this work is the one developed by Nandasana and Kumar [16] for lactic acid production by *Enterococcus faecalis* RKY1 and is as follows:

Specific growth rate:

$$\mu = \frac{\mu_{\max} S K_{iX}}{(K_{SX} + S)(K_{iX} + S)} e^{-(P/K_{pX})}. \quad (1)$$

The biomass production rate:

$$\frac{dX}{dt} = (\mu - K_d)X. \quad (2)$$

The substrate consumption rate:

$$\frac{dS}{dt} = -q_{s,\max} \frac{S K_{iS}}{(K_{SS} + S)(K_{iS} + S)} e^{-(P/K_{pS})} X. \quad (3)$$

The product production rate:

$$\frac{dP}{dt} = \alpha \frac{dX}{dt} + q_{p,\max} \frac{S K_{iP}}{(K_{SP} + S)(K_{iP} + S)} e^{-(P/K_{pp})} X. \quad (4)$$

For (3), the usual assumption proposed by Nandasana and Kumar [16] was not used, due to the nature of the process; a minus sign was included. According to the same authors, these assumptions are taken in consideration for the simplification of the modeling step:

(i) $K_{ss} = K_{sp}$, low lactose concentration affects in the same way lactose uptake and lactic acid production;

(ii) $K_{is} = K_{ip}$, high lactose concentration inhibits in the same way lactose uptake and lactic acid production;

(iii) $K_{ps} = K_{pp}$, lactate concentration affects in the same way lactose uptake and lactic acid production.

The method used to solve model differential equations for a given set of kinetic parameters was ODE113s solver of MATLAB [28], in order to obtain X, S, and P values. The initial conditions were the experimental values of X, S, and P at time zero ($t = t_0$).

2.4.2. Model Integration. In order to estimate kinetic parameters, a search of these parameters values, for which predicted values of X, S, and P are closer to the experimental one, needs to be applied. For this process, an optimization method was used. The nonlinear optimization method chosen for this problem was the interior-point approach to constrained minimization using the "fmincon" function in MATLAB [29, 30]. This function is a local search algorithm that starts with an initial guess and finds a constrained minimum.

The objective function (OF) used in this minimization was in the form of

$$\begin{aligned}
\mathrm{OF}_X &= \frac{\sum_{i=1}^{n} \left(X_i - X_{i,\mathrm{EXP}}\right)^2}{\sum_{i=1}^{n} \left(X_i - \overline{X_i}\right)^2} \cdot 100, \\
\mathrm{OF}_S &= \frac{\sum_{i=1}^{n} \left(S_i - S_{i,\mathrm{EXP}}\right)^2}{\sum_{i=1}^{n} \left(S_i - \overline{S_i}\right)^2} \cdot 100, \\
\mathrm{OF}_P &= \frac{\sum_{i=1}^{n} \left(P_i - P_{i,\mathrm{EXP}}\right)^2}{\sum_{i=1}^{n} \left(P_i - \overline{P_i}\right)^2} \cdot 100, \\
\mathrm{OF} &= \frac{\mathrm{OF}_X + \mathrm{OF}_S + \mathrm{OF}_P}{3}.
\end{aligned} \quad (5)$$

Thus, it was considered in the estimation of parameters that all variables are equally important and given equal weight to the approximation error for each one of them. The procedure to find the parameters was repeated at least 30 times, with random initial guesses, in order to assure a good solution.

3. Results and Discussion

3.1. Effect of Culture Media and Scale-Up on Lactic Acid and Biomass Productivity. This study is conducted to evaluate the effect of scale-up on growth and lactic acid production by *Enterococcus faecalis* SLT13. The fermentation was first carried out at laboratory scale, in 2 L bioreactor, than at semipilot scale, in 20 L bioreactor. All the studied fermentations were conducted in Stirred Tank Reactor (STR). Moreover, two distinct culture media were analyzed including the synthetic medium M17 and the papaïn Hydrolyzed Cheese Whey (HCW) in order to characterize the growth kinetics of *Enterococcus faecalis* SLT13. 2 L bioreactor cultures were conducted in M17 and HCW under controlled conditions, while only HCW was used for 20 L bioreactor; the same parameters (pH, temperature, and stirrer) were maintained.

The key kinetic parameters during the culture of *E. faecalis* in the conditions mentioned below are estimated experimentally and summarized in Table 1.

Whereas the final biomass is higher in synthetic media (M17), lactic acid concentration is more important when *E. faecalis* was grown on HCW. Lactic acid yield on lactose (Y_{ps}) increases significantly in HCW (from 0.314 to 0.98 g/g). Lactic acid yield obtained for the strain *E. faecalis* RKY1 based on sugar consumption varies from 0.90 to 0.99 for different initial sugar concentrations [2].

The ability of *Enterococcus faecalis* SLT13 to conduct lactose-to-lactic acid production is enhanced in hydrolyzed

Table 1: Main experimental data from the different batch cultures of *Enterococcus faecalis* SLT13.

Parameters	2 L M17	2 L HW	20 L HW
Biomass (g/L)	3.16	2.57	2.67
Lactic acid (LA) (g/L)	6.04	32.23	22.8
Y_{ps} (g/g)	0.314	0.98	0.72
Y_{xs} (g/g)	0.142	0.069	0.074
Volumetric productivity LA (g/L·h)	0.60	1.33	0.95

whey. This suggests that the carbon/nitrogen ratio in this kind of culture media is more favorable to lactic acid production than biomass growth. This fact is confirmed by the decrease of biomass yield on lactose (Y_{xs}), which suggests that the carbon source available in the medium is rather directed towards lactic acid production. Values of Y_{xs} are in the same range of magnitude than those reported for others species: *Enterococcus faecalis* CBRDO1 cultivated in LA5 medium supplemented with various concentration of glucose (5–20 g·L^{-1}) presented Y_{xs} from 0.03 to 0.06 g/g [31]. *Enterococcus faecalis* RKY1 grown on cane-sugar molasses had 0.07–0.19 g/g [2]. Ziadi et al. [21] presented values varying between 0.18 and 0.20 g/g for two strains of *Lactococcus lactis* SLT6 and SLT10 cultured on hydrolyzed cheese whey.

The volumetric lactic acid productivities are higher in HCW and reach 1.33 g/L·h in 2 L bioreactor. The ability of *E. faecalis* to produce L(+)- lactic acid from glucose or lactose by homolactic fermentation was earlier demonstrated in the literature. Lactate is generated through pyruvate reduction and two enzymes are involved in this reaction; the cytosolic NAD + -dependent L-(+)-lactate dehydrogenases: ldh-1 and ldh-2 [32].

Enterococcus faecalis is able to produce lactic acid from different substrates; Oh et al. [15] reported productivities higher than 0.8 g/L·h for *E. faecalis* RKY1 using barley and wheat as nutrient source.

While biomass is enhanced by scale-up, lactic acid production is negatively affected although the scale-up ratio 1 : 10 is respected. This decrease can be explained by many reasons. During industrial fermentation, scale-up causes changes in the geometry and consequently hydrodynamic of the bioreactor, especially for the STR. Two important changes occurred and can affect microbial survival conducting to a decrease of productivities: the mass transfer and the shear stress. The changed geometric conditions affect mixing behavior and consequently mass transfer which lead to a less availability of nutriments for the cells. The increasing shear stress during scale-up affects intrinsic resistance of the lactic-acid-producer microorganism [33].

Indeed, in mechanical agitated bioreactors and independently from microorganism type, the stirrer is the main dispersing tool allowing contact between both phases, biotic and abiotic, of the system. For an optimal process with optimal kinetic parameters, mass transfer between cells and culture media is crucial. Thus, choosing the appropriate stirrer speed and design is essential [34]. Several stirrer designs are available; the most used on microbial fermentation is the flat blade turbine type Rushton (containing 4, 6, or 8 blades) assuring a radial flow. This type of turbine is adapted to the agitation of Newtonian fluids such as culture media. However, shear stress caused by this type of turbine is important, particularly in the case of fragile microorganisms. Knowing that the impeller used in both 2 L and 20 L bioreactors consists of 2 and 3 turbine type Rushton, respectively, the decrease in lactic acid productivity during scale-up can be attributed to the high shear stress caused by this kind of turbine. The effect of shear stress on LAB was earlier demonstrated when studying the cell metabolism of *Lactobacillus delbrueckii* subsp. *bulgaricus*; cells cultivated at 72 Pa were affected by shearing forces; however, when cultivated at 36 Pa, metabolism was improved [35].

Thus, a good comprehension of kinetic behavior during bioreactor scale-up of lactic acid production is of crucial importance.

3.2. Estimation of Parameters. The experimental data obtained from batch fermentations of *Enterococcus faecalis* SLT13 on M17 and Hydrolyzed Cheese Whey in 2 L and 20 L bioreactors were used for developing the kinetic models and estimation of kinetic parameters. Results are presented in Table 2.

Differences in the maximum specific growth rate (μ_{max}) values obtained from the parameters estimation are observed. Higher growth rate is achieved when the strain is cultivated in HCW in 20 L bioreactor (1.09 h^{-1}). Similar values have been reported for *Enterococcus faecalis* RKY1 grown on cane-sugar molasses 1.6 h^{-1} [16]. The low growth rate in M17 was found previously for *Enterococcus faecalis* EF37 grown on the same medium [20]. *Enterococcus faecalis* CBRDO1 grown on LA5 medium supplemented with various concentration of glucose (5–20 g·L^{-1}) showed μ_{max} values of 0.59–0.64 h^{-1} [31].

The substrate limitation constants K_{sx}, K_{ss} and K_{sp} (Monod constants) for biomass production, sugar utilization and lactic acid production respectively are estimated. Although no effects of substrate limitation are observed for fermentation in HCW in 2 L bioreactor, substrate limitation of biomass is observed when *Enterococcus faecalis* SLT13 was cultured on HCW in 20 L bioreactor (the substrate limitation constant for growth of biomass K_{sx} is 4.229 g/L). Substrate limitation of sugar consumption is observed when the strain is cultured on M17 in 2 L bioreactor (the substrate limitation constant for sugar consumption K_{ss} was 2.73 g/L).

In this study, substrate inhibition is not observed; the key constants for substrate inhibition (K_{ix}, K_{is} and K_{ip}) are ≥100 g/L (Table 2). Meanwhile, initial substrate concentration does not exceed 45 g/L.

The product inhibition key constants (K_{px}, K_{ps} and K_{pp}) are also estimated and listed in Table 2. Although no substrate inhibition is observed, lactate inhibition of biomass growth occurs in HCW since the values of K_{px} are 3.77 and 5.00 g/L for 2 L and 20 L bioreactors respectively. The values of K_{px}, K_{pp} and K_{ps} reported for *Enterococcus faecalis* RKY1, cultured in molasses, were 17.074 and 29.1664 g/L respectively [16]. The inhibition by lactic acid can be caused by both undissociated and dissociated forms. Seen that lactic acid is completely dissociated at pH ≥ 6 and the fermentations occurs at pH 7, product inhibition is mainly due to completely dissociated form.

Table 2: Best-fitting values of kinetic parameters obtained by modeling *Enterococcus faecalis* SLT13 growth, sugar utilization, and lactic acid production in M17 and HCW in 2 L and 20 L bioreactors.

Kinetic parameters	2 L M17	2 L HCW	20 L HCW
Biomass production model			
μ_{max} (h^{-1})	0.34	0.99	1.09
K_{ix} (g/g)	114.06	399.75	394.20
K_{sx} (g/g)	0.023	0.0023	4.229
K_{px} (g/g)	18.11	3.77	5.001
K_d (h^{-1})	0.013	0.0001	0.0001
Sugar utilization model			
K_{is} (g/g)	290.13	399.99	143.391
K_{ss} (g/g)	2.73	0.01	0.15
K_{ps} (g/g)	10.44	11.16	20.07
q_{smax} (g/g·h)	4.92	4.99	4.16
Lactic acid production model			
K_{ip} (g/g)	135.89	100.00	373.89
K_{pp} (g/g)	5.31	44.99	42.83
K_{sp} (g/g)	0.025	0.01	0.065
q_{pmax} (g/g·h)	1.027	2.04	1.863
α (g/g)	0.052	0.01	0.017
R^2	94.34	88.81	97.87

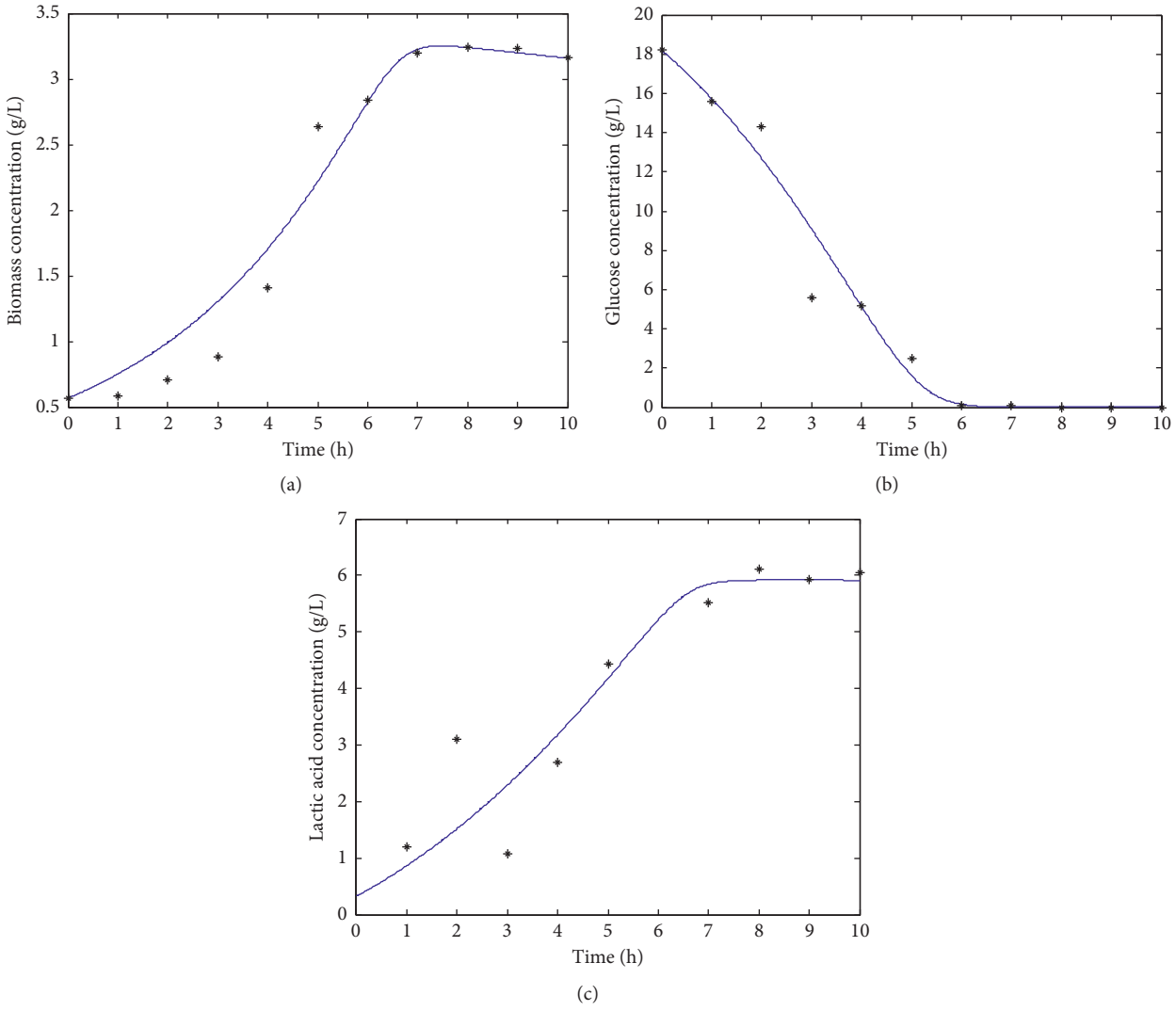

Figure 1: Experimental data (points) and simulation (lines) of biomass (a), glucose (b), and lactic acid (c) concentrations of 2 L bioreactor batch culture of *Enterococcus faecalis* SLT13 on M17 medium.

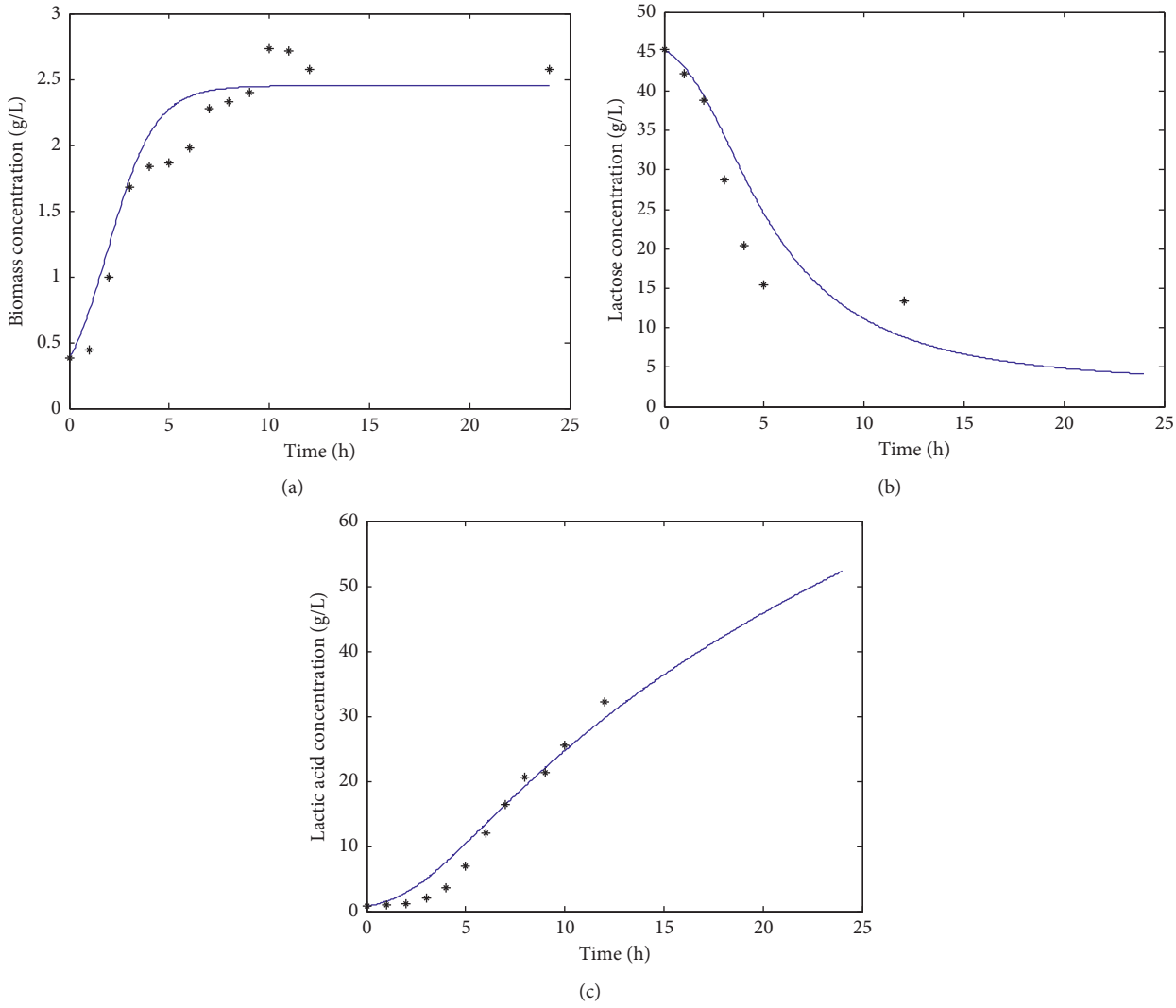

Figure 2: Experimental data (points) and simulation (lines) of biomass (a), lactose (b), and lactic acid (c) concentrations of 2 L bioreactor batch culture of *Enterococcus faecalis* SLT13 on hydrolyzed cheese whey.

For sugar consumption, the higher maximum sugar uptake rate ($q_s$max) is estimated to be 4.99 g/(g·h) for HCW in 2 L bioreactor, in spite of the values are close for the three fermentations. The maximum specific lactic acid production rate ($q_p$max) is higher for HCW and reach 2.04 g/(g·h) in 2 L bioreactor.

The growth-associated term (α) is very low for all the fermentations. In spite of that lactic acid production is both growth-associated and non-growth-associated and seen the value of α, the production of lactic acid is essentially occurred in the stationary phase. Higher growth-associated parameter (α) was reported by Nandasana and Kumar [16] for *Enterococcus faecalis* molasses and was 0.26 g/g. *Lactococcus lactis* NZ133 grown on lactose showed a highest value 0.36 g/g [36].

Evaluation of the cell death coefficient or endogenous decay constant (K_d) of *Enterococcus faecalis* is conducted for the three fermentations. The estimated decay constants are very low and equal to 0.0001 h^{-1} on HCW. Nevertheless, decay constant is relatively important for the fermentation in M17 and is equal to 0.013 h^{-1}. Death coefficient is strain dependent and different K_d values were reported in the literature. *Lactococcus lactis* SLT6 and SLT10 grown in the same media used in this study (HCW) presented values of 0.093–0.26 h^{-1}respectively [21]. *Enterococcus faecalis* RKY1 grown on cane-sugar molasses had K_d value of 0.00318 h^{-1} [16]. The lower K_d value for HCW can be ascribed to the protective effect of cheese whey proteins. Indeed, whey proteins are commonly used as protective media during the spray-drying and storage of lactic acid bacteria [37].

3.3. Comparison of Predicted and Experimental Parameters. The model used in this study and developed by Nandasana and Kumar [16] reflects closely the kinetic behavior of *Enterococcus faecalis* SLT13. The comparison of experimental data and predicted one is shown in Figures 1–3.

The value of correlation coefficient (R^2) is presented in Table 2 and is used to determine if the model fits well

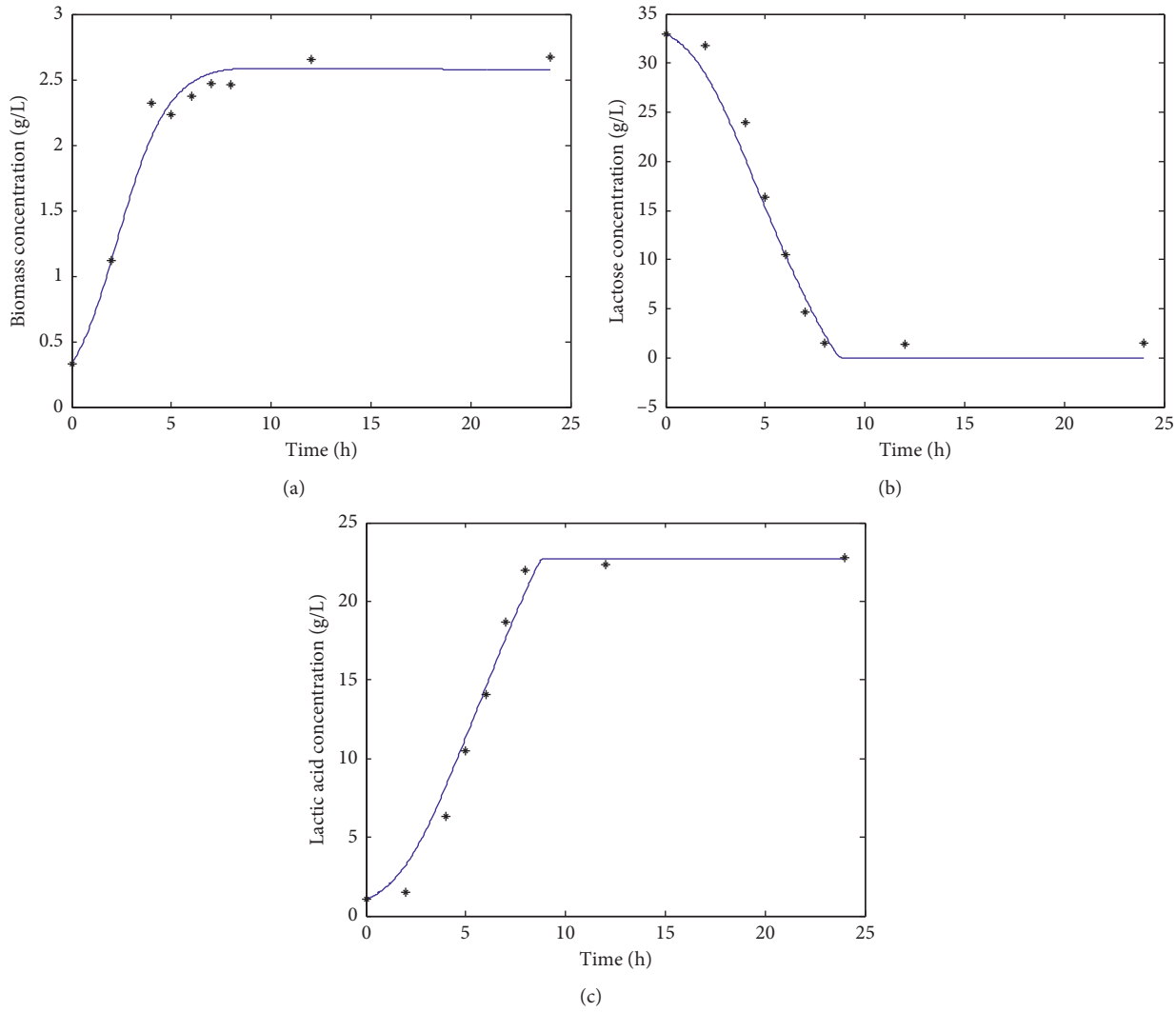

FIGURE 3: Experimental data (points) and simulation (lines) of biomass (a), lactose (b), and lactic acid (c) concentrations of 20 L bioreactor batch culture of *Enterococcus faecalis* SLT13 on hydrolyzed cheese whey.

the experimental data. A good linearity between model and experimental data is observed, especially for the fermentations occurring in 2 L M17 and 20 L HCW.

For all the fermentations, no lag phase is observed proving the ability of enterococci to adapt to different growth conditions. Exponential growth phase lasts 6 h when the strain was cultured in M17 followed by a short stationary phase. When cultured in HCW, exponential growth phase lasts only 5 h, while the stationary phase is relatively long. Glucose is totally consumed after 6 h of fermentation which confirmed that in M17 medium, the main factor affecting sugar utilization is substrate limitation (the substrate limitation constant for sugar consumption K_{ss} was 2.73 g/L). In HCW, while few quantities of lactose remain at the end of fermentation in 2 L bioreactor, it is almost totally consumed in 20 L bioreactor after about 10 h of fermentation (1.4 g/L) which confirms that the growth of biomass is limited by lactose concentration since K_{sx} is 4.229 g/L. Furthermore, the initial concentration of lactose is higher in 2 L bioreactor. Indeed, in cheese whey powder, concentration of lactose can vary from batch to batch.

In all fermentations, lactate production began earlier in the exponential growth phase and continues during the stationary phase. Indeed, lactic acid production is growth-associated as well as non-growth-associated. Lactic acid production remains constant after 7 h and 10 h of fermentation in M17 and in HCW in 20 L bioreactor, respectively. However, on HCW in 2 L bioreactor, lactate concentration continues to increase with the availability of the substrate.

4. Conclusions

The overall results obtained in our study show that cheese whey is suitable for lactic acid production; volumetric productivities are higher than the one obtained in M17. The mathematical modeling is useful and allows characterizing growth and lactic acid production of *Enterococcus faecalis* SLT13 in two different media and

two different volume bioreactors. The scale-up affects lactic acid productivity but enhances biomass growth. Substrate inhibition did not impact significantly the maximum lactic acid productivities attainable in the both 2 L and 20 L bioreactors. However, product inhibition seems to play a major role in the two studied media.

Abbreviations

K_d: Death coefficient (h^{-1})
K_{ip}: Substrate inhibition constant for lactic acid production (g/L)
K_{is}: Substrate inhibition constant for sugar consumption (g/L)
K_{ix}: Substrate inhibition constant for growth of biomass (g/L)
K_{pp}: Product inhibition constant for lactic acid production (g/L)
K_{ps}: Product inhibition constant for sugar consumption (g/L)
K_{px}: Product inhibition constant for growth of biomass (g/L)
K_{sp}: Substrate limitation constant for lactic acid production (g/L)
K_{ss}: Substrate limitation constant for sugar consumption (g/L)
K_{sx}: Substrate limitation constant for growth of biomass (g/L)
P: Lactic acid concentration (g/L)
$q_{p\max}$: Maximum specific lactic acid production rate (g/(g h))
$q_{s\max}$: Maximum specific sugar utilization rate (g/(g h))
S: Sugar concentration (g/L)
X: Biomass concentration (g/L)
$Y_{p/x}$: Lactic acid yield on growth of biomass (g/g)
$Y_{p/s}$: Lactic acid yield on sugar consumption (g/g)
$Y_{x/s}$: Biomass yield on sugar consumption (g/g).

Greek Symbols

α: Growth-associated constant in Luedeking–Piret model (g/g)
$\mu_{\max}$: Maximum specific growth rate (h^{-1}) of lactic acid
μ: Specific growth rate (h^{-1}).

Data Availability

All the numerical data used to support the findings of this study are available from the corresponding author upon reasonable request.

Additional Points

Kinetic modeling of lactic acid and biomass production by *Enterococcus faecalis* SLT13 was realized on synthetic media and Hydrolyzed Cheese Whey. Hydrolyzed Cheese Whey enhances lactic acid production. Scale-up from 2 L bioreactor to 20 L bioreactor increases biomass but not lactic acid production. Biomass growth and sugar utilization are affected by lactic acid inhibition in Hydrolyzed Cheese Whey.

Conflicts of Interest

The authors declare no financial or commercial conflicts of interest regarding the publication of this paper.

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Supplementary Materials

Graphical abstract representing lactic acid production by *Enterococcus faecalis* SLT13 during batch culture on Hydrolyzed Cheese Whey in 2 L and 20 L bioreactors. (*Supplementary Materials*)

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Research Article

Chemical Composition and Biological Activities of Extracts from Pomelo Peel By-Products under Enzyme and Ultrasound-Assisted Extractions

Pham Van Hung,[1] **Nguyen Hai Yen Nhi**,[1] **Ling Yu Ting**,[1] **and Nguyen Thi Lan Phi**[2]

[1]*Department of Food Technology, International University, Vietnam National University in Ho Chi Minh City, Quarter 6, Linh Trung Ward, Thu Duc District, Ho Chi Minh City, Vietnam*
[2]*Department of Food Technology, University of Technology-Vietnam National University in Ho Chi Minh City, 268 Ly Thuong Kiet Street, District 10, Ho Chi Minh City, Vietnam*

Correspondence should be addressed to Pham Van Hung; pvhung@hcmiu.edu.vn and Nguyen Thi Lan Phi; nglanphi@gmail.com

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Enzyme-assisted extraction (EAE) and ultrasound-assisted extraction (UAE) were popular methods used to extract bioactive compounds from citrus peels, by-products of fruit processing industry. In this study, the total phenolic content (TPC), total flavonoid content (TFC), naringin and hesperidin contents, and antioxidant and antimicrobial activities of the extracts from pomelo peels using the combined enzyme and ultrasound-assisted extraction (E-UAE) or ultrasound and enzyme-assisted extraction (U-EAE) technique were investigated and compared with those extracted using the EAE and UAE. The optimal EAE conditions were as follows: enzyme concentration of 2%, water-solid ratio of 40 ml/g, incubation temperature of 50°C, and extraction time of 60 min, whereas the optimal UAE conditions were ultrasonic energy of 40 kHz, water-solid ratio of 40 ml/g at room temperature, and extraction time of 60 min. The results indicate that the total phenolics, total flavonoids, naringin, and hesperidin contents of the extracts significantly increased in the following order of the extraction techniques: UAE < EAE < U-EAE < E-UAE ($p < 0.05$). The combined E-UAE technique was the most effective technique for bioactive compound extraction with the highest antioxidant and antimicrobial activities. The results also indicate that chemical composition and antioxidant and antimicrobial activities of the extracts were different depending on the pomelo species.

1. Introduction

Flavonoids, a group of pigments including flavones, flavanones, flavonols, isoflavones, anthocyanidins, and flavanols (or catechins), are present in dietary fruits and vegetables and responsible for flower and fruit coloration [1]. Flavonoids are well known for their health benefits such as antioxidative, anti-inflammatory, antimutagenic, and anticarcinogenic properties [1, 2]. Pomelo (*Citrus maxima*), a member of the citrus family, is a native plant in tropical and semitropical countries including Vietnam. People eat fresh pomelo directly or make juices; therefore, its peel usually becomes dump without recognizing possibly nutritional value even though the pomelo's peel holds 30% of the fruit weight [3]. Pomelo peel, a by-product of fruit processing, is a rich source of flavonoids [4, 5], which have anti-inflammatory and anticancer characteristics and play important roles in the prevention of cardiovascular disease, diabetes, and other diseases [6]. Therefore, pomelo peels could be inexpensively and readily available resources of bioactive compounds for application in the food and pharmaceutical industries [7]. The flavonoids found in pomelo almost are flavanones [4, 5]. Among them, naringin and hesperidin are the major compounds possessing strong antioxidant capacity, creating sweetness, and flavoring [4, 8, 9]. Nowadays, the ultrasound-assisted and enzyme-assisted extraction methods have been widely used for extracting bioactive compounds because they are considered as the innovation

green extraction technology without using toxic solvents [10]. The enzyme-assisted extraction (EAE) techniques have been shown to improve the extraction efficiencies of antioxidant compounds including phenolics, flavonoids, anthocyanins, and carotenoids and preserve their functional properties because plant cell walls are degraded and broken down by enzymes to release bioactive compounds [11]. Likewise, the ultrasound-assisted extraction (UAE) method has been widely used because of its higher extraction efficiency with shorter extraction time compared to traditional methods [12]. Until now, most of the studies on extraction of bioactive compounds from citrus peels used either EAE or UAE technique only [13]. Therefore, the objective of this study is to investigate the effects of combined ultrasound and enzyme treatment and vice versa on total flavonoids, naringin, and hesperidin concentration, antioxidant capacity, and antimicrobial activities of extracts from peels of different pomelo species.

2. Materials and Methods

2.1. Materials. Da Xanh, Nam Roi, and Tan Trieu pomelos (*Citrus grandis limonia* Osbeck species) grown in Tien Giang, Vinh Long, and Dong Nai provinces, respectively, were used in this study. The voucher specimens of the plant were confirmed by Dr. Nguyen Thi Lan Phi of the University of Technology, VNU-HCM (No. HCMUT201, HCMUT202, and HCMUT203). Fresh pomelo fruits were collected from those provinces in December 2017 and immediately transported to the laboratory to obtain their peels. After cleaning, the green parts of the peels were peeled off, cut into small pieces (approximately $1.0 \times 1.0 \times 1.0\ cm^3$ in dimension), and dried in the oven at 50°C for 24 h until the moisture content reached around 10%. The dried peel pieces were then ground and sieved to obtain fine powder by passing through a sieve of 0.105 mm in aperture size. The fine powder was then kept in a desiccator until use.

The Pectinex Ultra SP-L (CAS number: 9032-75-1), commercially purchased from Novozymes, was used in this study.

2.2. Extraction of Flavonoids from Pomelo Peels

2.2.1. Enzyme-Assisted Extraction. Enzyme-assisted extraction (EAE) was used in this study based on the method of Jeong et al. [14] with a slight modification. The dried pomelo peel fine powder (0.25 g) was accurately weighed and put into a test tube with 10 ml of distilled water, a green extraction solvent. A volume of Pectinex Ultra SP-L (0, 1, 2, 3, or 4%, v/w sample) was added to find out optimal enzyme concentration to extract flavonoids. The suspension was well shaken and then incubated in a shaking incubator at 50°C for 60 min. After incubation, the enzyme was deactivated by boiling the suspension at 90°C for 5 min, and then, the supernatant was carefully collected after centrifuging at 5,000 rpm for 10 min.

2.2.2. Ultrasound-Assisted Extraction. Ultrasound-assisted extraction (UAE) was done based on the method of Ma et al. [15] with a slight modification. Approximately 0.25 g of pomelo peel powder was accurately weighed and put into a test tube with 10 ml of distilled water. The solutions were well shaken using a vortex mixer. Then, the test tube was placed in a sonicator (WUC-A03, Daihan, India) with a frequency of 40 kHz at room temperature and treated for 30, 40, 50, and 60 min to determine the optimal time. After incubation, the test tube was then centrifuged at 5,000 rpm for 10 min at room temperature. The supernatant after centrifuging was quickly and carefully transferred into a new test tube and then stored in the refrigerator. All samples were carried out in triplicate.

2.2.3. Combined Extractions. In the combined method, EAE and UAE were combined in different order which was enzyme and ultrasound-assisted extraction (E-UAE) method or ultrasound and enzyme-assisted extraction (U-EAE) method. The samples were extracted by EAE [14] and then by UAE [15] named as enzyme and ultrasound-assisted extraction (E-UAE) and vice versa as ultrasound and enzyme-assisted extraction (U-EAE). All samples were carried out in triplicate.

2.3. Determination of Total Phenolic Content of the Extracts. The total phenolic content of extracts was determined using the Folin–Ciocalteu assay according to the report by Jeong et al. [14]. A mixture of 0.5 ml extract, 0.5 ml Folin–Ciocalteu reagent, and 1.0 ml saturated sodium carbonate was added into a test tube. Then, the volume was made up to 10 ml by distilled water and thoroughly mixed by the vortex. After incubation at room temperature in the dark for 45 min, the tube was centrifuged at 5,000 rpm for 5 min. The absorbance of the supernatant was measured with a spectrophotometer (Genesys 10S UV-Vis, USA) at 725 nm. The standard curve was prepared using gallic acid solution (20, 40, 60, 80, and 100 μg/ml) and expressed in milligram gallic acid equivalent per gram of sample (mg GAE/g). Total phenolic content was calculated by the following equation: TPC (mg GAE/g) $= (240.23 \times A_s \times \mathrm{DF})/W_s$, $R^2 = 0.9991$, in which A_s is the absorbance of the sample solution measured at 750 nm, DF is the dilution factor, and W_s is the mass of the sample.

2.4. Determination of Total Flavonoids Content of the Extracts. The total flavonoid content (TFC) was determined using the colorimetric method described by Hung and Morita [16] with a slight modification. A volume (0.5 ml) of each extract was mixed with 1 ml of 95% ethanol, 0.1 ml of 1 M potassium acetate, and 0.1 ml of 10% aluminum chloride solutions. Then, the volume was made up to 5 ml by distilled water. The tubes were thoroughly mixed using the vortex mixer and incubated at room temperature in the dark for 30 min. The absorbance of solutions was measured using a spectrophotometer (Genesys 10S UV-Vis, USA) at 415 nm. The standard curve was prepared using rutin solution and expressed in milligram rutin equivalent (RE) per gram of sample (mg RE/g). Rutin solution was diluted to 20, 40, 60, 80, and 100 μg/ml to form standard calibration. Total flavonoid content was calculated by the following equation:

TFC (mg RE/g) = $(165.2 \times A_s \times \mathrm{DF})/W_s$, $R^2 = 0.9934$, in which A_s is the absorbance of the sample solution measured at 415 nm, DF is the dilution factor, and W_s is the mass of the sample.

2.5. HPLC Analysis. Flavonoid compounds including naringin and hesperidin in pomelo peel's extracts were evaluated using a HPLC method [17] with slight modification. The extracts after freeze-drying for 3 days were diluted with 5 ml of the buffer including 0.02 M sodium acetate buffer (pH = 4) and methanol (1 : 1, v/v). Then, the mixture was filtered through a 0.45 μm membrane filter before injecting into a HPLC machine (Dionex Ultimate 3000 HPLC System, USA). The analysis was performed on a C18 column (150 mm × 4.6 mm, 5 μm particle size; Wakosil-II 5C18 HG, Japan) with degassed mobile phase consisting of two solvents: acetonitrile (A) and water (B) at a flow rate of 1 ml/min. The gradient elution was conducted as follows: 23% A in 8 min, 23–65% A in 7 min, 65–70% A in 5 min, 70–23% A in 1 min, and at 23% A in 1 min. The peaks were detected at 280 nm. Naringin (≥90%, from citrus fruit, code: N1376-25G) and hesperidin (≥80%, code: H5254-25G) purchased from Sigma-Aldrich Co. (Singapore) were used as standards.

2.6. DPPH Scavenging Analysis. The antioxidant activity of pomelo peel extracts was determined based on the method of Hung and Morita [16]. A volume of 0.1 ml extract was mixed with 3.9 ml of DPPH solution (0.075 mM). The mixture was kept in dark at room temperature for 30 min ($t = 30$). Then, the absorbance of the solution was measured immediately using a spectrophotometer (Genesys 10S UV-Vis, USA) at 515 nm. Blank was made by mixing the DPPH solution (3.9 ml) and 0.1 ml of water, and the absorbance was measured at the same wavelength immediately after mixing ($t = 0$). The scavenging of DPPH was calculated as follows:

$$\%\ \mathrm{DPPH\ scavenging} = \left[\frac{(\mathrm{Abs}\,(t = 0) - \mathrm{Abs}\,(t = 30))}{\mathrm{Abs}\,(t = 0)}\right] \times 100. \quad (1)$$

2.7. Determination of Antimicrobial Activities

2.7.1. Microbial Strains. There were four strains of bacteria used in this test, including *Staphylococcus aureus* (code: ATCC-6538 from Institute of Drug Quality Control, Ho Chi Minh), *Bacillus cereus* (code: VTCCB-1005 from Vietnam National University Institute of Microbiology and Biotechnology), *Pseudomonas aeruginosa* (code: ATCC-9027, from Institute of Drug Quality Control, Ho Chi Minh), and *Salmonella typhimurium* (code: ATCC-14028 Institute of Drug Quality Control, Ho Chi Minh). *S. aureus* and *B. cereus* are Gram-positive bacteria; *P. aeruginosa* and *S. typhi* are Gram-negative bacteria.

2.7.2. Diffusion Method. The agar diffusion method was used to evaluate the antimicrobial activity of extracts [18]. A 90 mm diameter Petri dish was filled with tryptone soybean agar (TSA, Himedia, India) to form a 3 mm thick agar plate. The bacteria culture (100 μl) was spread on the plate and 6 wells were created: 5 wells for 5 different extract solutions and 1 well for control (without extract). The extracts were diluted with sterilized distilled water with a ratio of 1 : 1, and then, 50 μl of each extract was added to the well. The control well was filled with 50 μl of solvent that was used to dilute extract. After incubation at 37°C for 24 h, the antimicrobial activity was evaluated by measuring the diameter of the inhibition zone in millimeter (including 6 mm diameter of the well) and compared with the control well.

2.8. Statistical Analysis. Analysis of variance (ANOVA) was used to analyze the data obtained from triplicate experiments to determine differences ($p < 0.05$) using the Statistical Package for the Social Sciences (SPSS).

3. Results and Discussion

3.1. Effect of Enzyme Concentration on Recovery of Flavonoid Content of Extract. The yields of total flavonoids extracted from pomelo peels under enzymatic treatment at different enzyme concentrations (0, 1, 2, 3, and 4%, v/w) are given in Figure 1. The total flavonoid content (TFC) increased with increasing enzyme concentration from 1 to 2% (v/w), and the TFC of the extracts with enzymatic treatments was significantly higher than that of the control without any treatment. However, the TFC of the extracts using enzyme concentration of 3 or 4% was significantly lower than that using enzyme solution of 2%. The previous study also reported that the enzymes were used to disrupt the pectin-cellulose complex in citrus peel and released higher flavonoids (naringin) in the extracts [19]. Nishad et al. [13] found that the optimized conditions for enzyme-assisted extraction of grapefruit peel were as follows: enzyme concentration of 0.9%, solvent-solid ratio of 40 ml/g, and extraction time of 4.81 h. In this study, the optimal extraction conditions were as follows: enzyme concentration of 2%, water-solid ratio of 40 ml/g, incubation temperature of 50°C, and extraction time of 60 min.

3.2. Effect of Ultrasonic Time on Recovery of Flavonoid Content of Extract. The yields of total flavonoids extracted from pomelo peels under ultrasound treatment are shown in Figure 2. With a frequency of 40 kHz at room temperature, the TPC of the extracts was significantly higher than that without ultrasound treatment. The TPC of the extract reached a peak at a treatment time of 50 min. Ma et al. [15] found that both ultrasonic time and temperature enhanced the total phenolic content of the extract. However, the temperature was the most sensitive on stability of phenolic compounds. In this study, the optimal ultrasound extraction conditions were ultrasonic energy of 40 kHz, water-solid ratio of 40 ml/g at room temperature, and extraction time of 60 min.

3.3. Bioactive Compounds of Extracts from Peels of Different Pomelo Species. Total phenolic, total flavonoid, naringin and hesperidin contents of the extracts from peels of different

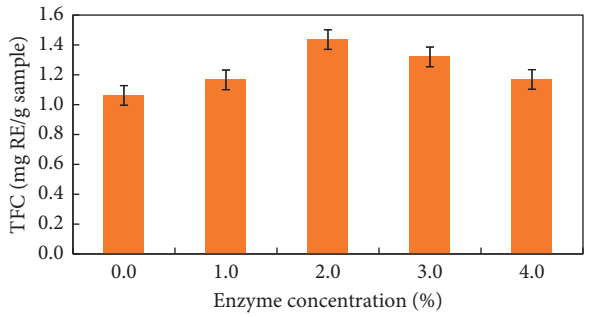

Figure 1: Effects of enzyme concentration on the recovery of total flavonoid content.

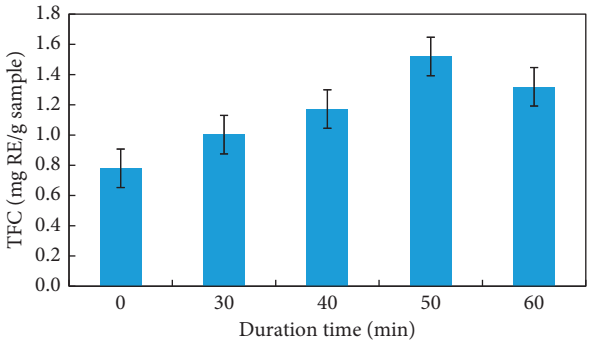

Figure 2: Effects of sonication time on the recovery of total flavonoid content.

pomelo species using ultrasound-assisted extraction (UAE), enzyme-assisted extraction (EAE), ultrasound and enzyme-assisted extraction (U-EAE), and enzyme and ultrasound-assisted extraction (E-UAE) techniques are shown in Table 1. Total phenolic, total flavonoid, naringin, and hesperidin contents of the extracts from pomelo peels were significantly affected by extraction techniques, in which the enzyme treatment had a higher impact than the ultrasound treatment. Nishad et al. [13] also reported that EAE was more effective than UAE in extracting bioactive compounds of *Citrus paradisi* L. peels, similar to the results of this study. The more effective action of enzyme in releasing flavonoid compounds than ultrasonic wave might be due to the selective breakdown of the cell walls by enzyme compared to the random breakdown of cell walls by ultrasound. The results also indicated that the combined ultrasound and enzyme treatments exhibited a significantly higher impact on total flavonoid, naringin, and hesperidin contents of the extracts than the individual enzyme or ultrasound treatment. As a result, the total phenolic, total flavonoid, naringin, and hesperidin contents of the extracts significantly increased in the following order of the extraction techniques: UAE < EAE < U-EAE < E-UAE ($p < 0.05$). The more efficient technique in extracting the flavonoid content of mulberry must (*Morus nigra*) is U-EAE compared to EAE or UAE, which was also reported by Tchabo et al. [20], because the cell walls were completely broken down after double treatments.

Among the three pomelo species, Tan Trieu pomelo peels contained the highest phenolic, flavonoid, and hesperidin contents, followed by Da Xanh and Nam Roi pomelo peels. The naringin content of the Da Xanh pomelo peels was significantly higher than those of the Nam Roi and Tan Trieu pomelo peels. These results indicated that the peels of different pomelo species had different chemical compositions. Even though Tan Trieu pomelo peels had the highest TFC, the naringin content of this sample was the least. The naringin and hesperidin contents of the peels of Tan Trieu pomelo extracted using the combined enzyme and ultrasound-assisted extraction in this study were 0.99 and 0.59 mg/g sample, consistent with the results reported by Wu et al. [21] for the hesperidin content of pomelo peel using the ultrasound-assisted extraction with ethanol as solvent.

3.4. Antioxidant Capacity of Extracts from Peels of Different Pomelo Species. The antioxidant activities of extracts from pomelo peels are given in Table 2. The extract from pomelo peels using the EAE was found to have higher antioxidant activity than that using the UAE. Likewise, the extract using the combined E-UAE had stronger antioxidant activity than that using the combined U-EAE. The results of antioxidant activities also proved that the combined E-UAE technique was the most effective technique for bioactive compound extraction with the highest antioxidant activity as compared to other techniques. Among the three pomelo species, the extract from Tan Trieu pomelo peels exhibited higher antioxidant activity than the others, followed by Da Xanh pomelo peels and Nam Roi pomelo peels. Thus, the results of antioxidant activities of the extracts from pomelo peels were positively correlated with the total flavonoid contents of these extracts. Actually, in some cases, the correlation was not clear because phenolic compounds are abundant while not all of them have the antioxidative ability. Therefore, the results in this study indicated that the extracts from pomelo peels might contain almost antioxidant compounds, resulting in the positive correlation between total flavonoid content and antioxidant activity results. Ru et al. [22] also reported that the flavonoid content and antioxidant capacity of the extracts from pomelo peels were significantly different depending on the extraction techniques. In this study, the combined enzyme and ultrasound extraction was shown to be more efficient than single treatment. In addition, the flavonoid content and antioxidant capacity of the extracts were affected by pomelo species and enzyme treatment conditions.

3.5. Antimicrobial Activities of Extracts from Peels of Different Pomelo Species. Antimicrobial activities of the pomelo peel extracts against *S. aureus, B. cereus, S. typhi*, and *P. aeruginosa* were determined by diffusion method, which measured the inhibition zone in of the well on agar plate (including 6 mm of the well), and are shown in Table 3. The results showed that the control extracts obtained by extracting pomelo peels without any treatment did not show any inhibition against *S. aureus, B. cereus, S. typhi*, and *P. aeruginosa* (data not shown). Using UAE, the extract of Da Xanh pomelo peels inhibited only the *B. cereus* and that of Tan Trieu pomelo peel only exhibited the *S. typhi*. In contrast, the extracts obtained from pomelo peels with enzyme

Table 1: Total phenolic, flavonoid, naringin, and hesperidin contents of extracts from pomelo peels using different extraction methods[1].

Type of pomelo	Total phenolics (mg GAE/g sample)	Total flavonoids (mg RE/g sample)	Naringin (mg/g sample)	Hesperidin (mg/g sample)
Ultrasound-assisted extraction				
Da Xanh	4.93 ± 0.32^{e}	1.54 ± 0.02^{ef}	1.09 ± 0.02^{c}	0.23 ± 0.01^{g}
Nam Roi	4.08 ± 0.27^{g}	0.93 ± 0.03^{i}	0.62 ± 0.02^{e}	0.13 ± 0.01^{i}
Tan Trieu	6.87 ± 0.28^{b}	1.90 ± 0.03^{c}	0.79 ± 0.02^{f}	0.49 ± 0.01^{d}
Enzyme-assisted extraction				
Da Xanh	6.70 ± 0.42^{b}	1.60 ± 0.01^{ef}	1.10 ± 0.09^{c}	0.20 ± 0.01^{f}
Nam Roi	5.65 ± 0.20^{e}	0.96 ± 0.02^{h}	0.68 ± 0.04^{e}	0.14 ± 0.01^{h}
Tan Trieu	7.38 ± 0.33^{b}	2.07 ± 0.01^{b}	0.85 ± 0.06^{e}	0.54 ± 0.01^{c}
Ultrasound and enzyme-assisted extraction				
Da Xanh	5.76 ± 0.06^{d}	1.55 ± 0.04^{e}	1.04 ± 0.09^{b}	0.27 ± 0.01^{ef}
Nam Roi	4.33 ± 0.08^{f}	1.09 ± 0.05^{gh}	0.89 ± 0.02^{de}	0.16 ± 0.01^{h}
Tan Trieu	7.06 ± 0.15^{b}	2.24 ± 0.05^{a}	0.87 ± 0.09^{de}	0.54 ± 0.01^{b}
Enzyme and ultrasound-assisted extraction				
Da Xanh	7.16 ± 0.12^{b}	1.76 ± 0.06^{d}	1.15 ± 0.07^{a}	0.29 ± 0.01^{e}
Nam Roi	6.03 ± 0.25^{c}	1.15 ± 0.04^{g}	1.04 ± 0.01^{d}	0.16 ± 0.01^{g}
Tan Trieu	7.50 ± 0.28^{a}	2.29 ± 0.05^{a}	0.99 ± 0.01^{d}	0.59 ± 0.01^{a}

[1]Values followed by the same superscript letters in the same column are not significantly different ($p < 0.05$).

Table 2: DPPH scavenging capacity (%) of extracts from pomelo peels[1].

Extraction methods	Da Xanh	Nam Roi	Tan Trieu
UAE	23.6 ± 1.0^{e}	13.7 ± 0.7^{g}	34.8 ± 1.1^{bc}
EAE	28.6 ± 1.1^{d}	23.2 ± 0.8^{e}	42.5 ± 1.0^{a}
U-EAE	25.8 ± 0.7^{e}	17.1 ± 0.6^{f}	36.9 ± 0.6^{b}
E-UAE	32.3 ± 0.5^{c}	24.0 ± 0.7^{e}	43.3 ± 1.6^{a}

UAE, ultrasound-assisted extraction method; EAE, enzyme-assisted extraction method; U-EAE, ultrasound and enzyme-assisted extraction method; E-UAE, enzyme and ultrasound-assisted extraction method. [1]Data (mean ± standard deviation) followed by the same superscript letters in the same column are not significantly different ($p < 0.05$).

Table 3: Antimicrobial activities of extracts from pomelo peels using different extraction methods[1].

Type of pomelo	Diameter of inhibition (mm)			
	S. aureus	*B. cereus*	*S. typhi*	*P. aeruginosa*
Ultrasound-assisted extraction				
Da Xanh	nd	10.7 ± 0.6^{a}	nd	nd
Nam Roi	nd	nd	nd	nd
Tan Trieu	nd	nd	8.3 ± 0.6^{a}	nd
Enzyme-assisted extraction				
Da Xanh	8.3 ± 0.6^{a}	10.7 ± 0.6^{a}	10.7 ± 1.5^{b}	10.3 ± 0.6^{b}
Nam Roi	12.3 ± 1.5^{c}	10.7 ± 0.6^{a}	10.0 ± 1.0^{b}	11.0 ± 1.0^{bc}
Tan Trieu	7.7 ± 0.6^{a}	10.0 ± 1.0^{a}	8.3 ± 0.6^{a}	8.0 ± 1.0^{a}
Ultrasound and enzyme-assisted extraction				
Da Xanh	8.7 ± 0.6^{a}	11.7 ± 0.6^{ab}	10.0 ± 2.0^{b}	10.7 ± 0.6^{b}
Nam Roi	11.7 ± 1.2^{bc}	11.3 ± 0.6^{ab}	9.3 ± 1.2^{ab}	10.7 ± 1.2^{b}
Tan Trieu	7.3 ± 0.6^{a}	11.3 ± 0.6^{ab}	10.3 ± 1.5^{b}	9.0 ± 1.0^{ab}
Enzyme and ultrasound-assisted extraction				
Da Xanh	10.7 ± 0.6^{b}	11.3 ± 0.6^{ab}	10.0 ± 2.0^{b}	8.0 ± 1.0^{a}
Nam Roi	12.3 ± 0.6^{c}	12.0 ± 0.1^{b}	11.3 ± 0.6^{c}	12.3 ± 0.6^{c}
Tan Trieu	10.7 ± 0.6^{b}	10.7 ± 0.6^{a}	10.3 ± 0.6^{b}	9.3 ± 1.5^{ab}

nd, not detected. [1]Data (mean ± standard deviation) followed by the same superscript letters in the same column are not significantly different ($p < 0.05$).

treatments (EAE, U-EAE, and E-UAE) exhibited positive antimicrobial activities against all types of bacteria. There was no significant difference in antimicrobial capacities of the extracts from pomelo peels obtained using the EAE and U-EAE techniques, while the stronger antimicrobial capacities of the extracts using the E-UAE were observed. The

results also indicated that the antimicrobial activities of the extracts from pomelo peels against four bacteria were not significantly different, while the extract of Nam Roi pomelo peels exhibited higher antimicrobial activities than the other pomelo species. The results in this study are consistent with the results of Abirami et al. [23], who reported that the crude extracts of pomelo peels using the conventional methanol extraction had antimicrobial activities of around 8 mm to 10 mm (including 5 mm paper disc) against *S. aureus, S. typhi*, and *P. aeruginosa*. Another research showed that the inhibition zone of the crude extracts using the conventional solvent extraction (dichloromethane, hexane, or ethyl acetate) was between 7.54 mm and 8.83 mm (including disc paper of 6 mm) against *S. aureus* [24]. Thus, the extraction techniques in this study using the combined enzyme and ultrasound treatment for extracting the bioactive compounds from the pomelo peels had more advantages with high antimicrobial activities and considered as the innovation green extraction technology without using toxic solvents.

4. Conclusion

In this study, enzyme-assisted and ultrasound-assisted extraction or their combination processes were carried out to extract the bioactive compounds from peels of three kinds of pomelos in Vietnam. The results indicated that the enzyme treatment had more impact on releasing the bioactive compounds than the ultrasound treatment. The E-UAE was found to be the most effective technique to obtain the extracts having the highest TPC, TFC, naringin, and hesperidin contents and antioxidant and antimicrobial activities compared to other extraction techniques. The results also indicated that Tan Trieu pomelo contains the higher TPC and TFC contents and antioxidant and antimicrobial activities than Da Xanh and Nam Roi pomelo species. Moreover, this research focused on "green" solution for the environment: using water as a solvent and taking all advantages of "waste."

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Hydrolysis Process Optimization and Functional Characterization of Yak Skin Gelatin Hydrolysates

Hui Yang,[1,2,3] Yanting Xue,[1,2,3] Jiaheng Liu,[1,2,3] Shunyi Song,[1,2,3] Lei Zhang,[1,2,3] Qianqian Song,[1,2,3] Li Tian,[1,2,3] Xiangyu He,[1,2,3] Shan He,[4] and Hongji Zhu[1,2,3]

[1]*Key Laboratory of Systems Bioengineering, Ministry of Education (Tianjin University), Tianjin 300072, China*
[2]*School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, China*
[3]*Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Tianjin 300072, China*
[4]*School of Chemistry and Chemical Engineering, Guangzhou University, Guangzhou, China*

Correspondence should be addressed to Hongji Zhu; zhj@tju.edu.cn

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Yak (*Bos grunniens*) is an animal mainly living on the Tibetan Plateau. Yak skin is a valuable resource that is wasted in the meat production process. This study aimed to prepare yak skin gelatin hydrolysates (YSGH) from yak skin through enzymatic hydrolysis and investigate functional characterization of YSGH. We showed that trypsin was more effective than neutrase, papain, and pepsin in increasing the degree of hydrolysis (DH) of YSGH. The conditions of enzymatic hydrolysis were optimized using central composite design (CCD) and response surface method (RSM), and the highest DH value of 31.96% was obtained. We then analyzed the amino acid compositions and molecular weight distribution of peptides in YSGH. The obtained YSGH exhibited certain antioxidant activity and excellent ACE-inhibitory activity (IC_{50} = 0.991 mg/mL). In addition, the solubility (98.79%), emulsification, and foaming properties of YSGH developed here were also evaluated. With these physicochemical and biological functions, YSGH had potential applications in food, pharmaceuticals, and cosmetics as an ingredient.

1. Introduction

Gelatin hydrolysates can be obtained by hydrolysis of gelatin from animal sources such as pigs [1], bovine [2], and fish [3]. It is reported that gelatin hydrolysates have different bioactivities such as antioxidant activities [4, 5], ACE-inhibitory activity [6, 7], antifreeze activity [8], and antiphotoaging activity [9]. Gelatin hydrolysates have been widely used in the production of pharmaceuticals and foods in the United States and Europe and have the potential for several advanced applications, such as smart drug delivery carriers for cancer therapy [10] and a new type of wound dressing [11]. Compared to gelatin, its hydrolysates are more easily to be absorbed. It was found that oral intake of gelatin hydrolysates has beneficial effects on skin recovery, including supporting wound closure and reducing skin wrinkles [12]. Oral intake of gelatin hydrolysates can also increase bone mass and prevent osteopenia [13]. In these previous studies, the functional properties of gelatin hydrolysates not only depended on the source of gelatin but also the types of enzymes and the enzymolysis conditions [14].

Due to the health concerns about consuming mammal and marine gelatin, there is a market for unpolluted animal gelatin-based products. With a population about 15 million around the world, yaks (*Bos grunniens*) live in a location where the altitude is about 3000 m above sea level, mainly along the border of China, India, and Nepal. In China, the population of yaks is the third largest among the cattle. During the evolutionary process, the yak's metabolism has been adapted to harsh living conditions such as high altitude and extreme cold. Living in the unpolluted places made yaks a preferable resource for nutrients and other bioactive products. By-products from different animal resources gained the spotlight with their potentials to be the raw material of bioactive compounds [15–18]. The leftovers from yaks processing, including the head, viscera, skin, and bone,

should be recycled and converted into value-added products. However, currently the wastes from yaks processing are usually discarded. It is not only causing environmental pollution but is also economically inefficient. Yaks processing wastes could account for up to 30.98% of the total weight, of which the majority is yak skin [13]. Yak skin consists of moisture (60%–70%), protein (30%–40%), fat (2%–4%), inorganic salt (0.5%–1.5%), and carbohydrates. The yak skin gelatin contains eighteen different amino acids, among which seven are essential amino acids and two are trace elements [19]. Therefore, yak skin can be an ideal raw material to produce gelatin hydrolysates by enzymatic hydrolysis. Previous study illustrated the possibility of extracting collagen and gelatin from meat by-products [20]. However, to date, little investigation has been done regarding optimal enzymatic hydrolysis process and the characterization of gelatin hydrolysates derived from yak skin.

Therefore, the objectives of this study were to optimize the bioprocess of enzymatic hydrolysis to produce gelatin hydrolysates from yak skin by using commercial protease and characterize the functionalities of the obtained gelatin hydrolysates.

2. Materials and Methods

2.1. Material. Yak skin was obtained from the yak market (Qinghai, China). Proteases of trypsin, neutrase, papain, pepsin, reagents of 1,1-diphenyl-2-picrylhydrazyl (DPPH), reduced L-glutathione (GSH), hydroxyproline, and the ACE synthetic substrate hippuryl-L-histidyl-L-leucine (HHL) were all purchased from Sigma-Aldrich (Shanghai, China). All reagents used in this study were of analytical grade.

2.2. Pretreatment of Yak Skin. Yak skin was soaked in water, and its impurities and hair were cleaned and removed, then chopped into $0.5 \times 0.5\ \text{cm}^2$ and stored at −20°C. The pieces of skin were mixed with n-butyl alcohol solution (1 : 10, w/v) at a ratio of solid to solution 1 : 20 (w/v) to remove fat and noncollagen protein. The mixture was stirred for 24 h at 4°C, then washed with distilled water until neutral pH. Defatted residues were treated with 0.1 M sodium hydroxide solution at a sample/alkaline solution ratio of 1 : 30 (w/v) with stirring for another 36 h at 4°C. Finally, the deproteinised skin was rinsed with water until it reached a pH of 7.0.

2.3. Extraction of Gelatin. The pretreated yak skin was rinsed with 0.2% HCl (w/v) solution (1 : 8, w/v) for 4 h at room temperature followed by rinsing with water until it reached a pH of 7.0. The residues were then soaked in distilled water (85°C) until the skin was completely dissolved in the solution. The supernatant was collected by centrifugation at $6580 \times g$ for 15 min at room temperature, then concentrated by rotary evaporator and lyophilized by a freeze drier (Alpha1-2, Christ, Germany). The freeze-dried powder, named was gelatin, was stored in a desiccator at room temperature until use. The gelatin yield was calculated by the ratio of freeze-dried powder to the raw material. Crude protein, lipid, and ash contents in the extracted gelatin were analyzed according to national text standard of China (GB/T 5009.5-2010, GB/T 5009.6-2003, and GB/T 5009.4-2010).

2.4. Enzymatic Hydrolysis. The type of enzyme plays an important role to the quality of DH. In order to identify the most efficient enzyme to produce gelatin hydrolysates from yak skin, enzymatic hydrolysis was performed using four proteases individually: neutrase (pH 7.0, 45°C), pepsin (pH 2.0, 37°C), trypsin (pH 7.5, 50°C), and papain (pH 6.2, 25°C) at each optimum condition with protease concentration of 2000 U/g. The optimum pH and temperature conditions for each enzyme are shown in the brackets above. After hydrolysis were processed with the selected time, the resulted hydrolysates solution was inactivated by boiling in water for 15 min, followed by centrifugation ($6580 \times g$, 15 min). The supernatants were collected to measure the DH and then lyophilized by a freeze drier (Alphal-2, Christ, Germany). The freeze-dried powder, which was named as gelatin hydrolysates, was stored in a desiccator at room temperature until use. For each enzyme, the gelatin hydrolysates with the highest DH were chosen to measure their DPPH scavenging activity.

2.5. Degree of Hydrolysis (DH). DH was determined by the ratio of the number of cleaved peptide bonds to the total number of bonds per unit mass weight. The degree of gelatin hydrolysis was evaluated according to the trinitrobenzene sulfonic acid (TNBS) method [21]. All determinations were made in duplicate. DH was defined as follows:

$$\text{DH}(\%) = \frac{(L_S - L_0)}{(L_{\max} - L_0)} \times 100, \quad (1)$$

where L_s is the content of free α-amino groups in the hydrolysate, L_0 is the content of free α-amino groups in gelatin, and $L_{\max}$ is the content of α-amino in substrate reacted with 6 mol/L HCL for 24 h at 100°C.

2.6. Experiment Design and Data Analysis

2.6.1. Fractional Factorial Designs of Experiments. Factorial design was carried out to screen 5 variable factors (pH, temperature, ratio of enzyme to substrate (E/S), substrate concentration, and the hydrolysis time). The aim of the factorial design was to identify relatively important variables and interactions among independent variables. The regression analysis of the variables was performed using SPSS software version 20.0 (IBM, USA).

2.6.2. Central Composite Design (CCD) and Response Surface Methodology (RSM) of Experiments. The enzymatic hydrolysis conditions were optimized by RSM based on single factor experiments and factorial designs. A CCD design with 3-factor and 3-level was applied to explore the effect of the independent variables on DH. Analysis of variance (ANOVA) was estimated with Design Expert software (Version 8.0.6, State-Ease Inc. Minneapolis, USA). All the

experiments were conducted in triplicate, and the average values were recorded as the response values with deviations.

2.7. Analysis of Physiochemical Properties

2.7.1. Amino Acid Analysis. The gelatin hydrolysates (10 mg) were hydrolysed in 5 ml of 6 M HCl at 110°C in a set of time in a vacuum and then neutralized with 3.5 M NaOH. The solution was diluted with 0.2 M citrate buffer (pH 2.2) after neutralization. Finally, the amino acids of the gelatin hydrolysates in the solution were identified and quantified by the automatic amino acid analyzer (Biochrom 30+, Pharmacia Biotech, UK).

2.7.2. Determination of Solubility. The nitrogen solubility index (NSI) was used to show the solubility of protein hydrolysates. In brief, gelatin hydrolysates (0.5 g) were dissolved in 50 ml of 0.1 M NaCl at pH 7.0 followed by centrifugation (640 ×g, 30 min). The nitrogen content in the supernatant was analyzed for nitrogen by the macro-Kjeldahl method [22]. NSI was calculated as follows:

$$\text{NSI}\,(\%) = \frac{A}{B} \times 100, \tag{2}$$

A is the nitrogen content of the supernatant and B is the total nitrogen content of the sample.

2.7.3. Emulsifying Properties. The emulsification activities (EA) and emulsification stability (ES) were determined as described by Shahidi et al. [23]. The gelatin hydrolysate sample (0.5 g) was dissolved in 25 ml distilled water (pH 7). Adding 25 ml of oil into the prepared gelatin hydrolysate solution, the mixture was transferred into 50 ml cylinders and homogenized at a speed of 10280 ×g for 2 min at room temperature. The obtained emulsion was divided into two portions. One was centrifuged at 230 ×g for 5 min. EA was calculated by the following equation:

$$\text{EA}\,(\%) = \frac{V_1}{V_0} \times 100, \tag{3}$$

where V_1 is the height of the emulsion layer and V_0 is the height of the mixture solution.

The other portion was incubated in water at 50°C, and the volume of the emulsion phase was recorded every hour. ES was calculated according to the following equation:

$$\text{ES}\,(\%) = \frac{V_2}{V_3} \times 100, \tag{4}$$

where V_2 is the total volume of the emulsion every hour and V_3 is the initial volume of the emulsion.

2.7.4. Foaming Properties. Foam expansion (FA) and foam stability (FS) were determined according to the method described by Shahidi et al. [23]. In brief, 0.5 g dried gelatin hydrolysates were dissolved in 50 ml distilled water, and then homogenized at 10280 ×g for 2 min at room temperature. The sample stood for 0, 1, 3, and 10 min. Meanwhile, the volume of the solution was recorded. FA and FS were calculated by the following equations:

$$\begin{aligned} \text{FA}\,(\%) &= \frac{A - B}{B} \times 100, \\ \text{FS}\,(\%) &= \frac{A_t - B}{A - B} \times 100, \end{aligned} \tag{5}$$

where A is the total volume after whipping, B is the original volume before whipping, and A_t is the total volume after standing for different lengths of time (0, 1, 3, and 10 min).

All measurements were carried out in triplicate.

2.8. Analysis of Biological Properties

2.8.1. Determination of Antioxidant Activities

(1) Determination of DPPH Scavenging Activity. The DPPH radical scavenging assay was performed according to the method reported by Nazeer et al. [24] with some modifications. The sample was mixed with ethanolic DPPH (0.1 mmol/L) at volumetric ratio of 1 : 1. The mixture was left in the dark for 30 min, and the absorbance was measured at a wavelength of 517 nm. The DPPH was calculated using the following equation:

$$\begin{aligned} &\text{DPPH radical scavenging activity}\,(\%) \\ &= \frac{A_{\text{control}} + A_{\text{blank}} - A_{\text{sample}}}{A_{\text{control}}} \times 100, \end{aligned} \tag{6}$$

where A_{control} is the absorbance of the control (distilled water instead of sample) and A_{blank} is the absorbance of the sample with ethanol instead of DPPH.

(2) Determination of Superoxide Anion Radical Scavenging Activity. Superoxide anion scavenging activity was measured using the methods described by Xie et al. [25] with some modifications. 0.2 mL sample, 4 mL distilled water, and 4.5 mL Tris-HCl buffer (0.05 mol/L; pH 8.2) were mixed together and incubated for 10 min at 25°C. 0.3 mL pyrogallol was added after incubation. The absorbance was measured at a wavelength of 299 nm every 30 s for 5 min. The superoxide radical scavenging activity was calculated using the following equation:

$$\text{scavenging anion activity}\,(\%) = \left(1 - \frac{A_i}{A_0}\right) \times 100, \tag{7}$$

where A_0 is the absorbance of the control (distilled water instead of sample) and A_i is the absorbance of the sample.

(3) Hydroxyl Radical Scavenging Activity. The scavenging capacity for hydroxyl radicals was measured according to the modified method described by de Avellar et al. [26]. The mixture, containing 0.2 ml *o*-phenanthroline (0.75 mM), 0.4 ml of 0.2 M phosphate buffer (pH 7.4), 0.2 ml distilled water, and 0.2 ml of 0.75 mM $FeSO_4$, was reacted with 0.2 ml H_2O_2 (0.1%, v/v) and a 0.4 ml sample at 37°C for 60 min. The absorbance of the resulting solution was measured at a

wavelength of 536 nm. The hydroxyl radical scavenging activity was calculated using the following equation:

$$\text{hydroxyl radical scavenging activity}\,(\%) = \frac{A_S - A_P}{A_B - A_P} \times 100, \tag{8}$$

where A_S is the absorbance of the control (distilled water instead of sample), A_B is the absorbance of the samples (distilled water instead of H_2O_2), and A_P is the absorbance of the samples.

2.8.2. Angiotensin-Converting Enzyme (ACE) Inhibitory Assay. The ACE-inhibitory effect was determined by the spectrophotometric method with some modifications [27]. A sample solution (50 μl) and 150 μl of 2.5 mM ACE synthetic substrate HHL reacted with 50 μl ACE (25 mU/ml) at 37°C for 1 h. The reaction was stopped by adding 1 M HCl (150 μl). The resulting hippuric acid was extracted by adding 1.5 ml ethyl acetate and followed by centrifugation (2570 $\times g$, 15 min). The hippuric acid was dissolved in 3 ml distilled water, and the absorbance was measured at a wavelength of 228 nm using a TU-1901 UV-spectrophotometer (Beijing, China). The ACE-inhibitory effect was calculated as follows:

$$\text{ACE-inhibitory activity}\,(\%) = \frac{A_a - A_b}{A_a - A_c} \times 100, \tag{9}$$

where A_a is the absorbance of the control, A_b is the absorbance of the sample, and A_c is the absorbance of the blank without ACE or the sample.

2.9. Statistical Analysis. All the experiments were carried out in triplicate. The results were recorded as means ± standard deviation and subjected to one-way analysis of variance (ANOVA) using SPSS software version 20.0 (IBM, USA). The significance was evaluated statistically by the F value at a probability (P) below 0.05.

3. Results and Discussion

3.1. Preparation of Yak Skin Gelatin. The composition of gelatin was related to the type of animal and the environment in which the animal grows. The flowchart of technological process was shown in Figure 1. The obtained gelatin contained protein (96.58%), lipid (1.27%), and ash (1.90%). The gelatin yield reached 52.97%, which is much higher than the reported extraction rates of many other animal gelatins [28–30]. The reason might be that yak skin contained more proteins and less lipids since yaks live in high altitudes with extremely cold climates [31]. Thus, the higher gelatin yield ensured the feasibility of its use in preparing gelatin hydrolysates.

3.2. Screening of Efficient Enzyme. DH value was generally used to evaluate the hydrolysis effectiveness of macromolecule proteins [32]. A higher DH value could represent the more numbers of short-chain peptides in the hydrolysates. Different proteases might exhibit different catalytic activity on yak skin gelatin due to their different specific catalytic centers. Therefore, four types of proteases including trypsin, neutrase, papain, and pepsin were applied in this study. The results of enzymatic hydrolysis of yak skin gelatin using these enzymes with an activity of 2000 U/g for 7 h, respectively, were shown in Figure 2. Based on DH value, the order of efficiency of the four enzymes was found to be the following: trypsin > neutrase > papain > pepsin. The highest DH value of 20.43% was attained with trypsin at 4 h. Trypsin, a serine endopeptidase, acts on the peptide linkage between the carboxyl groups of lysine and arginine. Its effectiveness has also been verified in enzymatic hydrolysis of fish skin, such as salmon [33] and flatfish [5].

3.3. Optimization of Enzymatic Parameter. The variables and coded levels were presented in Table S1. The experiment design and results were shown in Table S2. DH ranged from 8.47% to 26.48% with different levels of factors. This obviously indicated that the variables of digestion could directly affect DH. The F value was 4.261, and the p value was 0.053 (Table S3). According to the regression analysis of variables shown in Table S4, the factors of temperature, E/S, and substrate concentration were found to have great effects on the hydrolysis reaction, among which substrate concentration was the most significant factor ($p = 0.006$). Therefore, these three factors were chosen for response surface analysis.

Based on factorial analysis, the enzymatic parameters were optimized by RSM. The CCD with 3-factor and 3-level was utilized to explore the effect of independent variables on DH (Table S5). The analysis of the developed quadratic polynomial model for variables was shown in Table S6. The value of coefficient determination R^2 was 0.8562, higher than 0.85. This indicated that the model was accurate and acceptable. According to the regression analysis, the variability in the response could be explained by the second-order polynomial model given below:

$$\text{DH} = 30.63 + 1.49C - 1.98D - 1.42B^2 - 1.07C^2 - 2.07D^2. \tag{10}$$

The equation was significant with a p value less than 0.01 (Table S6). DH of hydrolysates was primarily determined by the linear and quadratic terms of the temperature, E/S, and substrate concentration. Among these factors, the most significant one was substrate concentration ($p < 0.001$).

The three-dimensional (3D) response surface plots (Figure 3) explained the results of statistical and mathematical analysis of the effects of temperature, E/S, and substrate concentration on DH. A quadratic relation was apparent between DH and the three variables. The value of DH, predicted by the Design Expert software program, reached its maximum by a combination of coded levels at 0.26 (B), 0.70 (C) and −0.48 (D). The corresponding variables were temperature of 51.32°C, E/S of 3695.45 (U/g) and substrate concentration of 6.3% (w/w), with the predicted response of DH being 31.72%.

In order to validate the above prediction, experiments were carried out using the predicted variables (shown

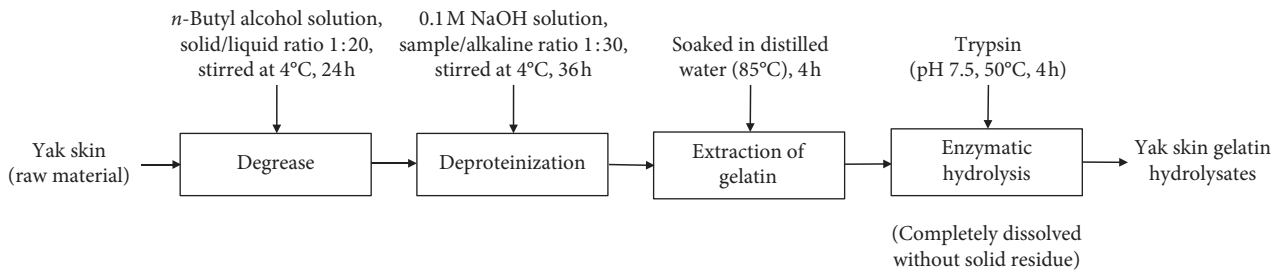

Figure 1: The technological process diagram of preparation of yak skin gelatin hydrolysates.

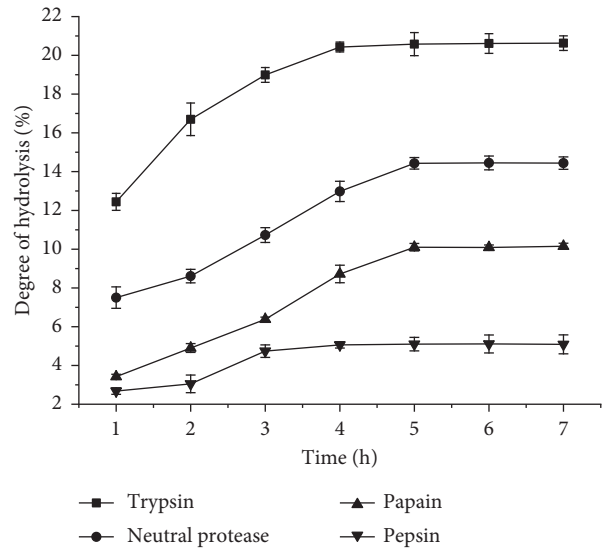

Figure 2: The degree of hydrolysis (DH) of yak skin gelatin hydrolysates by different enzymes at 2000 U/g and hydrolysis times. Results are displayed as average ± standard deviations from three replications.

above), with slight adjustments: temperature of 51°C, E/S of 3695 (U/g), and substrate concentration of 6.3% (w/w). The DH of the resulted gelatin hydrolysates reached 31.96%. Compared with the predicted number of 31.72%, relative error was only 0.75%. This experiment validated the accuracy of the experimental design in this study. And the DH increased from 20.43% to 31.96% under optimized conditions.

3.4. Amino Acid Composition and Molecular Weight Distribution of Peptides in YSGH. Amino acid composition of the protein hydrolysates were influenced by protein source, type of protease, and hydrolysis conditions, and played an important role in the physiochemical and biological properties of hydrolysates. Thus, we detected the amino acid composition of the YSGH. As shown in Table 1, YSGH contained a considerable amount of glycine (19.87 ± 0.24%), proline (12.87 ± 0.40%), glutamate (10.34 ± 0.11%), hydroxyproline (7.08 ± 0.56%), and alanine (6.50 ± 0.17%). These amino acids have been proved to be essential for the functions of many bioactive peptides, such as antioxidant activity [35–37], ACE-inhibitory activity [38], and antimicrobial activity [39]. Furthermore, large amounts of hydrophilic amino acids (65.18%), as well as the high DH of YSGH (31.96%), ensured the solubility of YSGH (98.79%). Additionally, flavor amino acids such as aspartate and glutamate participated in flavor development of the products [40]. Thus, YSGH is expected to have excellent biological properties and have potential to be used as source of functional peptides in food industry.

Meanwhile, we analyzed the molecular weights of peptides in YSGH by GPC (gel permeation chromatography). The molecular weights of the peptides were mainly distributed in the range of 400 to 3500 Da. The peptides in the molecular weight range of 1000-2236 Da accounted for the largest proportion of components (Table 2). Furthermore, many researchers have found that the peptides in this range of molecular weight showed excellent biological activities, such as antioxidant activity [41] and ACE-inhibitory activity [7].

3.5. Emulsifying and Foaming Properties. As shown in Table S7, YSGH exhibited a certain degree of emulsifying activity (47.6 ± 0.7%) and emulsion stability ranging from 91.7 ± 0.5% to 79.1 ± 0.3%. ES decreased slightly within 5 h. The hydrolysates with short-chain peptides showed acceptable solubility and various hydrophobic groups. It is assumed the amphiphilic polymers with hydrophobic and

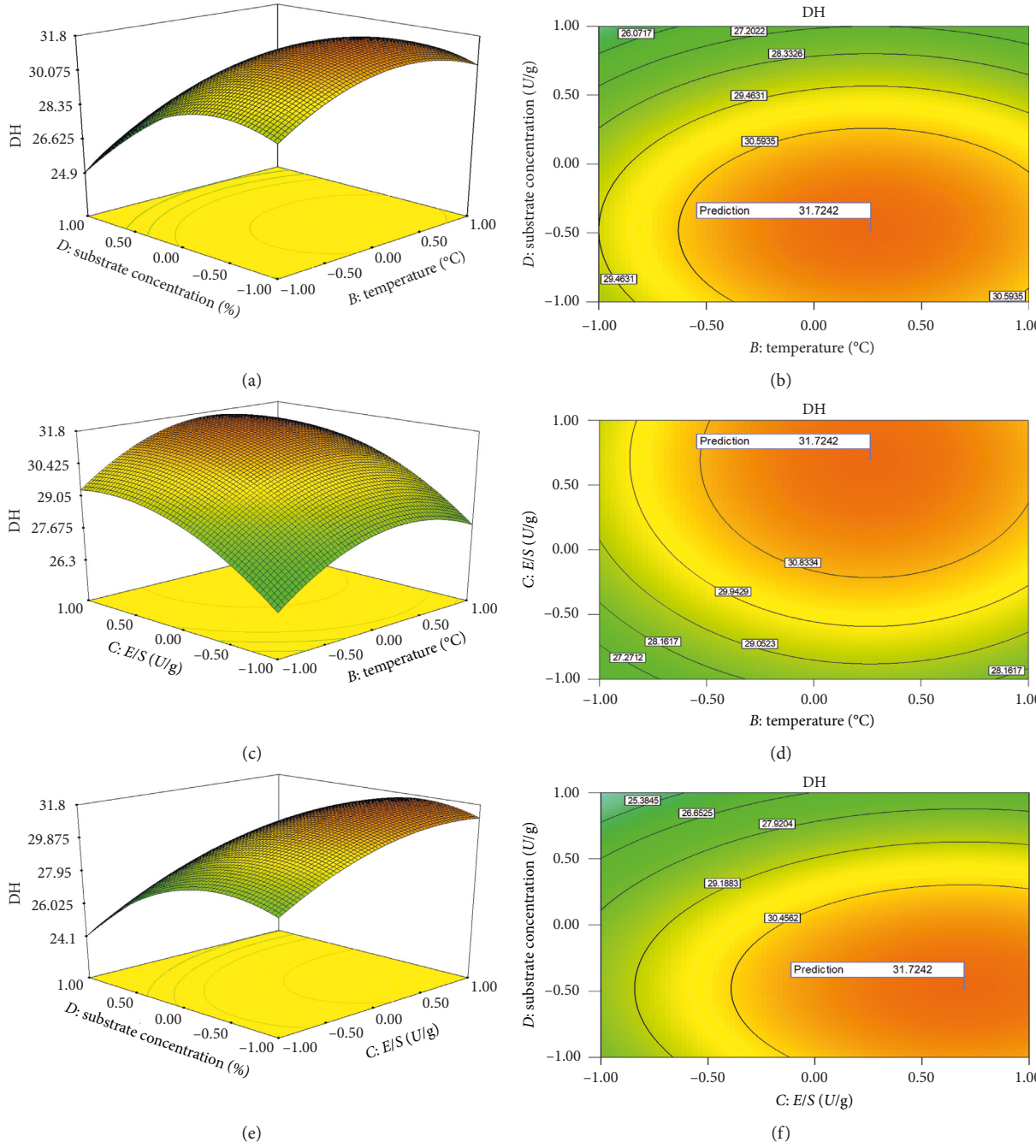

Figure 3: Response surface three-dimensional map and contour plots of the degree of hydrolysis.

hydrophilic moieties tended to protect oil in water with homogenization and film formation. Hence, the hydrolysis process can increase the hydrophilic groups and also expose the hydrophobic groups at the surface. This phenomenon leads to the formation of amphipathic complexes to reduce surface tension and stabilize the surface film [42]. On the other hand, the high degree of solubility contribute to the rapidly hydrolysates diffuse and allow absorption at the interface. Moreover, trypsin treatment contributes to the emulsifying properties [43, 44]. Due to its outstanding emulsifying properties, YSGH could be used as an emulsifier in the food industry.

Foaming property is an important functionality of collagen peptides and often used in food products. The foam capacity (FC) and foam stability (FS) of YSGH is presented in Table S8. The stability drops rapidly in the first ten minutes but becomes more stable with the time increase. For foam formation, the hydrolysates should be soluble in liquid and be capable of rapid migration and orientation to form an interfacial film that can decrease the surface tension. Since

Table 1: Amino acid composition of yak skin gelatin hydrolysates and porcine skin gelatin hydrolysates.

Amino acid	Composition/100 g gelatin hydrolysates of yak skin [34].
Aspartate	4.90 ± 0.38
Threonine	1.35 ± 0.16
Serine	2.32 ± 0.31
Glutamic acid	10.34 ± 0.11
Proline	12.87 ± 0.40
Glycine	19.88 ± 0.24
Alanine	6.50 ± 0.17
Cysteine	0.46 ± 0.02
Valine	1.86 ± 0.07
Methionine	0.98 ± 0.04
Isoleucine	1.08 ± 0.09
Leucine	2.40 ± 0.19
Tyrosine	1.02 ± 0.02
Phenylalanine	2.34 ± 0.25
Histidine	0.63 ± 0.03
Lysine	3.02 ± 0.32
Arginine	5.73 ± 0.23
Hydroxyproline	7.08 ± 0.56
Hydrophilic amino acid	55.25 ± 1.56 (65.18%)
Hydrophobic amino acid	29.51 ± 0.48 (34.82%)
Total	84.76 ± 2.02

Values are given as means ± standard deviations from triplicate determination.

Table 2: The molecular weight distribution of yak skin gelatin hydrolysates.

Molecular weight distribution (MW)	Weight percentage (w%)
198–416	4.96
418–996	22.43
1000–2236	42.46
2245–3502	20.11
3516–5442	6.95
5465–16640	3.09

simultaneous dehydration and hydrophobic portions of the hydrolysates are favorable for thermodynamics, the spontaneous adsorption of hydrolysates from solution to the air/aqueous interface is a major driving force for foaming formation [45]. The hydrolysis of gelatin can shorten the amino acid chain and reduce the surface tension resulting in parcels of gas bubbles and to improve foam stability. YSGH, a hydrolysis product with many hydrophobic regions, exhibited the certain degree of foaming properties. Surface hydrophobicity had been reported to have effective relations with foaming properties [46].

3.6. Biological Properties of YSGH

3.6.1. Antioxidant Activities. Antioxidants have important roles both in food and in human body by counteracting oxidation processes. Recently, an increasing number of researches focused on exploring the antioxidants content of foods, especially of the animal by-product wastes [47]. In this study, DPPH scavenging activity, superoxide anion radical scavenging activity, and hydroxyl radical scavenging activity were evaluated, respectively, and the results were compared with reduced glutathione (GSH), which is a commercial antioxidant. As shown in Figure 4(a), the DPPH scavenging activity of YSGH increased linearly with the hydrolysates concentration. When the concentration of YSGH was 5 mg/ml, the DPPH-scavenging activity of YSGH reached 59.79%, higher than that of flatfish skin hydrolysates [5] and *Pseudosciaena crocea* protein hydrolysates [48]. As expected, the DPPH-scavenging activity of YSGH is much higher than that of porcine skin gelatin hydrolysates as well (19.25%) [34]. These results were in accordance with previous studies, which showed that hydrolysates and peptides isolated from bovine skin gelatin owned antioxidant properties [49]. In addition, many researches demonstrated that the peptides with lower molecular weight exhibits higher antioxidant activity. It is well known that the types of enzymes and enzymatic hydrolysis conditions could influence the molecular weight distribution and functional properties of the hydrolysates [14]. Thus, the trypsin hydrolysates showed the highest DPPH scavenging activity, which positively correlated with the high DH (Table 3).

Superoxide anion radical, as a main radical resource in *vivo*, can produce hydrogen peroxide and hydroxyl radicals that can lead to cytotoxicity. Figure 4(b) showed that the superoxide anion radical scavenging activity of YSGH was maintained at 28.19% while the concentration ranged from 1 mg/ml to 5 mg/ml, indicating that YSGH exhibited a certain degree of superoxide anion radical scavenging activity.

Scavenging of hydroxyl radicals plays an indispensable role in the body. The hydroxyl radical scavenging activity of YSGH was approximately in direct proportion to the concentration of YSGH and reached its maximum (53.28 ± 1.46%) at 5 mg/ml (Figure 4(c)) ($p < 0.05$). It was reported that antioxidant properties of hydrolysates depend on amino acid composition, structure, and hydrophobicity. Above all, YSGH exhibited great antioxidant activities against DPPH, superoxide, and hydroxyl radicals, indicating that YSGH has great potential in being an antioxidant against oxidative damage.

3.6.2. ACE-Inhibitory Activity. The ACE-inhibitory activity acts as a major role in the control of blood pressure. Usually, ACE-inhibitory peptides have been reported to be short peptides with Pro residues. It has been reported that the presence of Leu residues has a positive correlation with both antioxidant and ACE-inhibitory activities [4]. The ACE-inhibitory activity of YSGH increased with the increase of concentration from 0 to 4 mg/ml (Figure 5). The ACE-inhibitory activity of YSGH shows a higher ACE-inhibitory activity ($IC_{50} = 0.991$ mg/ml) than that of bovine skin gelatin hydrolysates treated with trypsin ($IC_{50} = 1.044$ mg/ml) [50]. YSGH exhibited great biological properties because it had a high DH by trypsin treatment, thus the molecular weight distribution of caused peptides become broader with more small peptides relating to the ACE-inhibitory activity. Also, a high positive correlation was found between ACE-inhibitory

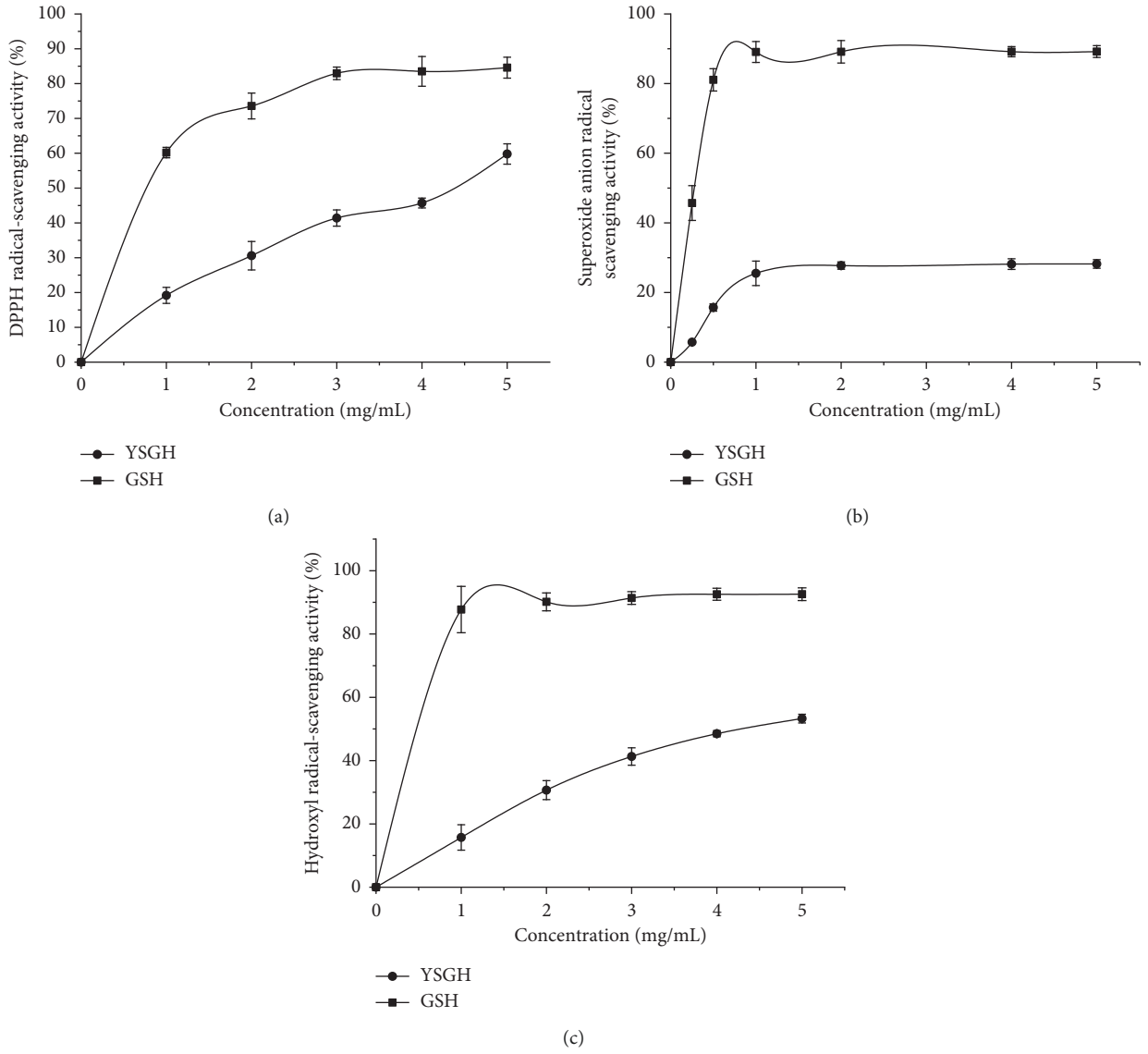

Figure 4: Scavenging effect of yak skin gelatin hydrolysates (YSGH) and reduced L-glutathione (GSH). (a) Effect on DPPH free radicals; (b) effect on superoxide anion radicals; (c) effect on hydroxyl free radicals. Data displayed as average ± standard deviations from three replications.

Table 3: DPPH scavenging activity of yak skin gelatin hydrolysates by four-enzyme treatment under each optimum conditions.

	Trypsin	Neutrase	Papain	Pepsin
DPPH scavenging activity (%)	53.22 ± 0.25	45.75 ± 0.34	39.55 ± 0.12	32.94 ± 0.09

Values are given as mean ± SD from triplicate determinations.

and DPPH radical scavenging activities in Alcalase hydrolysates of soya protein [49].

4. Conclusion

This study developed an economic and efficient process for preparing bioactive YSGH from yak skin through enzymatic hydrolysis. In general, the bioavailability of gelatin products is influenced by the molecular weight distribution and amino acid composition, which was associated with the DH of the hydrolysates. Trypsin hydrolysates showed the highest DH and DPPH scavenging activity compared to those attained by neutrase, papain, and pepsin.

The optimum conditions for preparing YSGH by trypsin were as follows: temperature of 51°C, E/S of 3695 (U/g), and substrate concentration of 6.3% (w/w). Under such conditions, the maximum DH value of 31.96% was attained, which agreed well with that predicted by the RSM model (31.72%). The obtained YSGH contained large amounts of hydrophilic amino acids (65.18%), and the peptides in the molecular

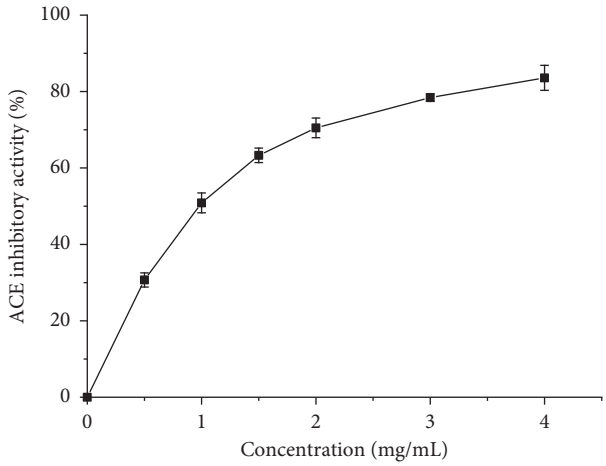

Figure 5: Effect on ACE-inhibitory activity. Data displayed as average ± standard deviations from three replications.

weight range of 1000–2236 Da accounted for the largest proportion of components. YSGH exhibited good results for the properties of solubility (98.79%), emulsifying and foaming, which makes it possible to be a functional food ingredient. More importantly, YSGH showed certain antioxidant activities and excellent ACE-inhibitory activities (IC_{50} = 0.991 mg/ml). Therefore, the YSGH prepared in this study should be of potential utility as a bioactive ingredient in health food and pharmaceutical industries.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Supplementary Materials

Supplementary Table S1: coded levels of the independent variables for fractional factorial designs used for yak skin gelatin hydrolysis. Table S2: program and results of the fractional factorial designs used for yak skin gelatin hydrolysis. Table S3: analysis of variance (ANOVA) for fractional factorial designs of DH. Table S4: regression equation for fractional factorial designs of DH. Table S5: levels of independent variables for DH (degree of hydrolysis) of yak skin gelatin hydrolysates and the results from response surface model. Table S6: analysis of variance (ANOVA) of developed quadratic polynomial model of DH. Table S7: emulsifying properties of yak skin gelatin hydrolysates. Table S8: foaming properties of yak skin gelatin hydrolysates. Figure S1: gel permeation chromatography of yak skin gelatin hydrolysis. (*Supplementary Materials*)

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