Utilization of orange pulp and corn steep liquor for L-methioninase production by *Wickerhamomyces subpelliculosus*

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Background and objective
L-methioninase has attracted much attention with respect to its proposed applications in both pharmaceuticals and food industry. The aim of this study was to develop an economic medium formulation using agro-industrial by-products as substrates for large-scale production of L-methioninase.
Materials and methods
Identification of a high L-methioninase-producing yeast isolate was carried out using 18S rRNA molecular technique. Screening of various agro-industrial by-products and optimization of different process parameters were investigated. Partial purification and characterization of a crude enzyme were carried out.
Results and discussion
A high L-methioninase-producing yeast isolate was phylogenetically identified as *Wickerhamomyces subpelliculosus*. Among different agro-industrial by-products tested, orange pulp supported maximum enzyme production (94.08 U/ml) followed by cane and beet molasses. In addition, corn steep liquor (CSL) gave high enzyme level (141.12 U/ml) and could be used as an inexpensive alternate for yeast extract. The optimum growth conditions were found to be orange pulp 30% (w/v), CSL 4% (v/v), $CaCl_2$ 0.05%, and KH_2PO_4 0.05% (w/v) at pH 6.0 after 48 h of incubation. This developed medium formulation increased L-methioninase production (161.95 U/ml) by twofold compared with that obtained by the Czapek–Dox's medium (73.92 U/ml). Crude enzyme was partially purified by heat treatment at 70°C with 2.9 purification fold. The enzyme activity was optimal at temperature 60°C and pH 7.0. The results showed that a mixed formulation of orange pulp and CSL can be used as an effective and economic substrate for the production of L-methioninase by *W. subpelliculosus*.

Keywords:
L-methioninase, production, orange pulp, *Wickerhamomyces subpelliculosus*

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Introduction

L-methioninase is a pyridoxal 5′-phosphate-dependent enzyme that catalyzes the direct conversion of L-methionine to methanethiol, α-ketobutyrate, and ammonia [1,2]. It has been found in several microorganisms including bacteria, yeast, and fungi [3–5].

Recently, L-methioninase has attracted much attention with respect to its proposed applications in both pharmaceuticals and food industry. It is one of few microbial enzymes with high therapeutic value as it has been explored to be an effective drug target for the treatment of methionine-dependent tumors (breast, lung, colon, kidney, and glioblastoma) [6–8]. In addition, L-methioninase has been exploited as a promising drug target for antibacterial, antifungal, and antiprotozoa therapies [9,10].

The exploitation of agro-industrial by-products for biotechnological applications in particular for the production of enzymes and other value-added fine products would be of greater significance in the future. They usually have a composition rich in sugars, minerals, and protein; therefore, they should not be considered 'wastes' but raw material for other industrial processes [11–13]. Regarding by-products from food industry, the interest has mainly focused on dairy, sugar industries (whey and molasses), and fruit-processing wastes (orange peel, apple pomace, and guava fruit residue). Microorganisms that have been used for this purpose include yeasts, fungi, and bacteria [14–16].

Production of enzymes is hindered by high manufacturing costs involved because the synthetic media turn out to be expensive and hence less preferred for large-scale production of enzymes. To build a sustainable and economically microbial process,

the use of expensive substrates, low productivity, and high pretreatment costs must be overcome. Approximately 30–40% of production cost is estimated to be the cost of the growth medium, particularly contributed by carbon and nitrogen sources [17]. Agro-industrial by-products and their complex organic contents constitute excellent and cheap sources of proteins and carbohydrates which serve as a rich media for microbial growth. Therefore, efforts are being made to develop organic carbon and nitrogen supplements from agro-industrial by-products to replace more costly sucrose, yeast extract, and peptone [18,19]. The previous publications on the production of L-methioninase have focused on the use of a synthetic medium [2,4,5]; however, there are no studies reported the complete replacement of commercial carbon and nitrogen sources with agro-industrial by-products.

Considering these facts, the aim of this study was to optimize various media components and process parameters for large-scale production of L-methioninase using agro-industrial by-products as carbon and nitrogen sources. Furthermore, partial purification and characterization of L-methioninase were carried out.

Materials and methods

Agro-industrial by-products

Sugarcane and beet molasses were supplied by Al-Hawamedia Sugar Company. Corn steep liquor (CSL) was obtained from maize products industries (Giza). Apple pomace and guava fruit residue were supplied by Guhayna Company, Six October Governorate. Oranges were collected freshly from the local market. All the chemicals used throughout this study were of analytical grade and procured from Sigma-Aldrich (St Louis, Missouri, USA).

Yeast strain identification

The yeast strain used in this study was isolated from a soil sample collected from Bani-Seuf Governorate, Egypt. The culture was maintained on malt yeast peptone slants at 4°C [20]. A high L-methioninase-producing strain was identified using 18S rRNA-based molecular technique. DNA extraction was done using protocol of Gene Jet Plant Genomic DNA Purification Kit (Thermo-Scientific, K0791, made in Germany), Qiagen, Valencia, USA. Primers used for PCR and DNA sequencing are ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCT TATTGA TATGC-3′). The PCR conditions were as follows: initial denaturation at 96°C for 3 min followed by 25 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 30 s and an extension step at 72°C for 1 min [21]. The purified PCR product was sequenced, and these sequences were subjected to BLAST similarity analysis available from NCBI Gen Bank database [22]. A phylogenetic tree was constructed using the Tamura-neighbor joining method by MEGA software, version 5.0 (Molecular Evolutionary Genetics Analysis, Bioinformatics, Tokyo Metropolitan University, Hachioji, Tokyo, Japan) [23].

Preparation of substrates

Orange pulp was prepared according to the method described by Aggelopoulos *et al.* [24]. In brief, the external parts of orange skins (yellow exocarp) were removed and then the whole remaining fruit was blended for 10 min and stored at −20 before use. The blended product of the whole fruit (not including juice) is hereinafter referred to as 'orange pulp.' Other substrates, such as cane and beet molasses, CSL, sugar syrup, cheese whey, apple pomace, and guava fruit residue, were used without any pretreatment [25,26].

Inoculum preparation

For inoculum preparation, the yeast strain was grown in modified Czapek–Dox's medium with the following composition (g/l): glucose, 20; yeast extract, 2.0; K_2HPO_4 1.0; $MgSO_4.7H_2O$, 0.5; and KCl, 0.5. The culture was incubated on rotating shaker at 30°C, 150 rpm for 24 h.

L-methioninase production

L-methioninase production was carried out in 250-ml Erlenmeyer flasks containing 15 g of defrosted orange pulp (wet weight basis) supplemented with 2% (v/v) CSL. The substrates were enriched with a salt solution with the following composition (% w/v): 0.1 KH_2PO_4; 0.05 $MgSO_4$; and 0.05 KCl, and then sterilized at 120°C for 15 min. The production medium was inoculated with 1 ml of inoculum (1×10^8 CFU/ml) and incubated with shaking (150 rpm) for 48 h. The medium composition varied with the experimental design. After the incubation period, the biomass was harvested by centrifugation (4500 rpm, 10 min), suspended in 50 mM sodium phosphate buffer (pH 6.5), and grounded according to the method described by Arfi *et al.* [27]. The cell-free extract was used as the crude enzyme.

L-methioninase assay

L-methioninase activity was determined according to the method described by Ferchich *et al.* [28], using L-methionine as a substrate. The reaction mixture

contained 20 mM L-methionine in 50 mM potassium phosphate buffer (pH 7.0), 0.01 mM pyridoxal5′-phosphate, 0.25 mM DTNB, and the crude enzyme in a final volume of 1 ml. The reaction mixture was incubated for 10 min at 50°C and released methanethiol from a substrate was detected spectrophotometrically at 412 nm. One unit of L-methioninase was expressed as the amount of enzyme that releases 1 µM of methanethiol per minute. The protein content of the samples was determined according to the method of Bradford [29]. The specific activity is expressed as units of L-methioninase per milligram protein.

Biomass determination

Yeast growth was determined based on cell dry weight. Samples of yeast culture (20 ml) were centrifuged at 4000 rpm for 10 min, dried at 105°C to constant time, and weighted.

Screening of different agro-industrial by-products for enzyme production

Various agro-industrial by-products (cane and beet molasses, CSL, sugar syrup, whey, orange pulp, apple pomace, and guava fruit residue) were screened as substrates for L-methioninase production. Overall, 15 grams of each substrate (orange pulp, apple pomace, and guava fruit residue) was transferred separately to 250-ml Erlenmeyer flasks, each containing the following nutrients (% w/v): 0.1 KH_2PO_4, 0.05 $MgSO_4$, and 0.2 yeast extract as a nitrogen source. Other substrates were directly used in the production medium after dilution. Czapek–Dox's medium was used as a control. The contents of the flasks were mixed and sterilized at 121°C and 1.5 atmospheric pressure for 20 min. The sterilized media were inoculated with 1% of inoculum and incubated with shaking at 30°C for 48 h.

Optimization of different process parameters

Different nutritional and cultural conditions influencing yeast growth and L-methioninase production were optimized as the effect of incubation period (24–48 and 72 h), orange pulp concentration (5, 10, 15, 20, and 25 g/50 ml medium), different organic (yeast extract, peptone, casein, malt extract, and methionine), and inorganic nitrogen sources (ammonium chloride, ammonium nitrate, ammonium phosphate, and ammonium sulfate). Furthermore, supplementation of the growth medium with different concentrations of CSL ranged from 2 to 7% (v/v) was also evaluated.

The optimum medium pH for L-methioninase production was determined in the presence of orange pulp and CSL as substrates using 50 mM of sodium citrate buffer or potassium phosphate buffer (4.0–8.0). In addition, the effect of different metal salts, MgSO4, KCl, KH_2PO_4, and $CaCl_2$ at two concentrations (0.05 and 0.1% w/v) on enzyme production was studied.

Partial purification of L-methioninase

The crude enzyme was partially purified by heat treatment as described by Selim *et al*. [19]. The enzyme activity and protein content of each fraction were measured.

Effect of temperature and pH on L-methioninase activity

The optimal temperature for L-methioninase activity was determined by carrying out the enzyme reaction at different temperatures from 30 to 75°C under optimal assay conditions.

The effect of pH on the enzyme activity was studied by varying the pH of the reaction mixture using 50 mM sodium citrate buffer (pH 4.0–6.5) and 50 mM potassium phosphate buffer (pH 6.5–8.0) at 60°C for 10 min.

Statistical analysis

All experiments were performed in triplicates, and the results are represented as mean±SD [30].

Results and discussion

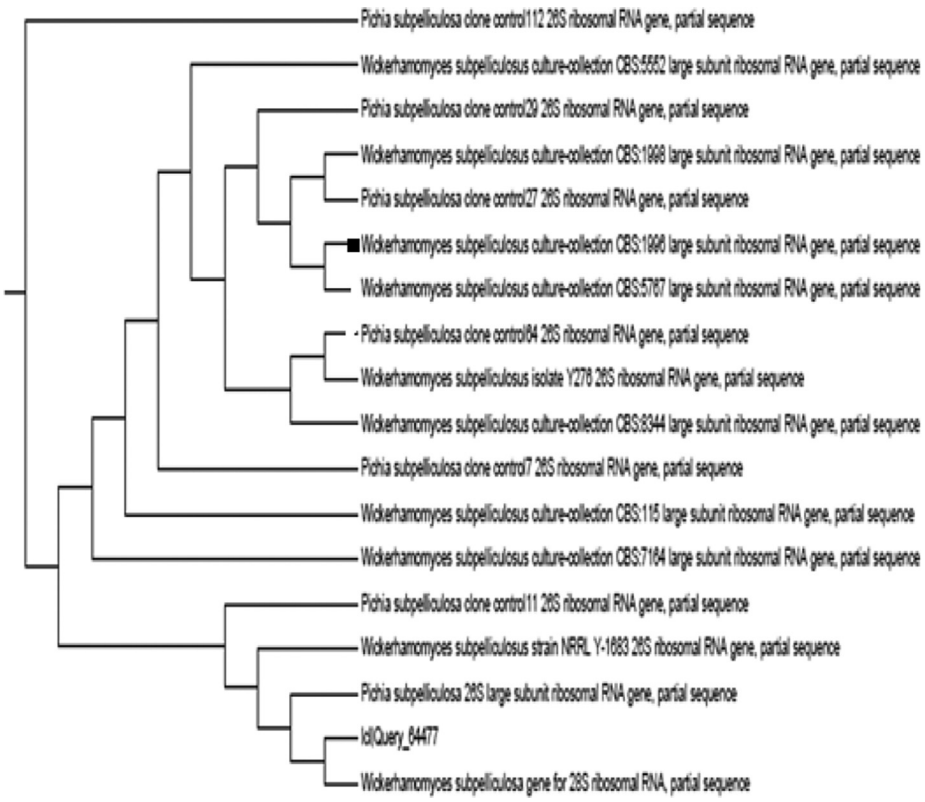
Identification of the yeast isolate

Molecular identification of a high L-methioninase-producing yeast strain was carried out based on the 18S rRNA molecular technique to reveal the phylogenetic relationship with other yeast species. As per the phylogenetic tree's suggestion, the yeast isolate is closely related to *Wickerhamomyces subpelliculosus* (Fig. 1). Therefore, *W. subpelliculosus* is proposed as its name.

Screening of various agro-industrial by-products for L-methioninase production

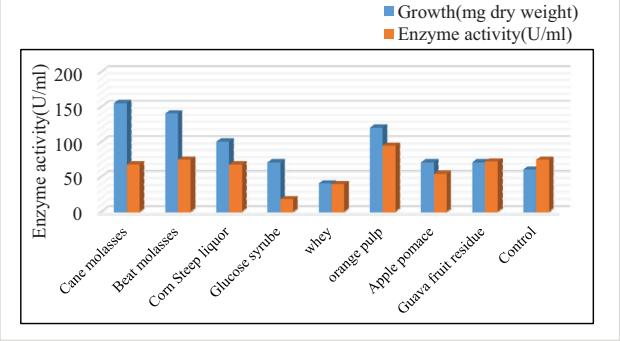
The selection of a suitable agro-industrial by-product for L-methioninase production depends on its nutrient content. Therefore, various agro-industrial by-products (cane and beet molasses, CSL, sugar syrup, whey, orange pulp, apple pomace, and guava fruit residue) were screened as the substrates for L-methioninase production by *W. subpelliculosus*. The level of enzyme production varied widely depending on the nature of the agro-industrial by-products as shown in Fig. 2. All the tested substrates except cheese whey supported good growth. However, the highest activity of L-methioninase was achieved in media containing orange pulp followed by cane and beet

Figure 1



Phylogenetic tree based on 18S rRNA gene sequences, showing the position of *Wickerhamomyces subpelliculosus* constructed by neighbor joining method.

Figure 2



Screening of different agro-industrial by-products for L-methioninase by *Wickerhamomyces subpelliculosus.*

molasses and CSL (94.08, 73.92, 67.20, and 67.0 U/ml, respectively). The superior effect of natural substrates for enzyme production may be owing to the presence of growth promoters in enough amounts covering the requirements of yeast growth [31]. In this respect, the utilization of orange pulp as a rich media for yeast growth was mainly owing to its high level of fermentable carbohydrates, which come from naturally occurring simple sugars (glucose and fructose) and polysaccharides, along with amino nitrogen [26,32]. On the contrary, molasses are rich in total sugars mainly sucrose and contain some amount of proteins and minerals, so that they serve as ideal substrates for the growth of yeast [33].

Optimization of L-methioninase production

Incubation period

The optimum incubation period for L-methioninase production was determined in the presence of the most favorable agro-industrial by-products (orange pulp and cane and beet molasses). As shown in Table 1, similar growth levels were obtained in the presence of cane molasses and orange pulp (173.0 and 172.50 mg, respectively). However, the highest enzyme level and specific activity were achieved after 48 h of incubation when orange pulp was used as a substrate (99.50 U/ml and 37.82 U/mg protein). In agreement with these results, the highest L-methioninase production by several cheese-ripening yeasts was reported after 48 h of incubation [27,34]. In addition, Mantzouridou *et al.* [26] obtained the highest yeast growth in media containing orange peel within 48 h of incubation.

Effect of different concentrations of orange pulp

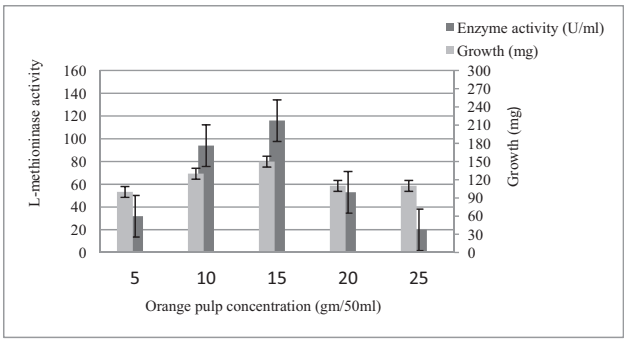
Further experiment was performed to determine the optimum concentration of orange pulp for maximum L-methioninase production (Fig. 3). Results show a

Table 1 Effect of different incubation periods on growth and L-methioninase production by *Wickerhamomyces subpelliculosus*

Agro-industrial by-products	Growth (mg)			Enzyme activity (U/ml)			Specific activity (U/mg protein)		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Orange pulp	156±1.40	172.5±3.53	171.5±2.12	62.46±0.91	99.5±0.70	80.57±0.90	31.02	37.85	32.26
Cane molasses	131±1.40	173±0.42	170±0.70	38.59±3.25	55.8±2.99	63.8±2.86	13.85	16.39	20.96
Beet molasses	142±2.50	152±2.50	157±2.50	48.5±1.50	73.19±0.61	75.91±1.99	24.0	22.12	28.21

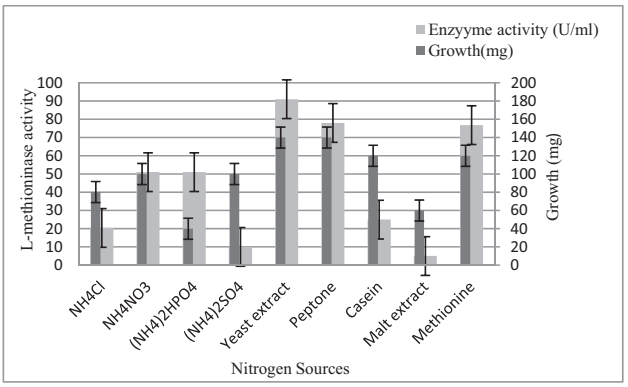
Data is expressed as mean±SD of triplicates.

Figure 3



Effect of orange pulp concentration on growth and L-methioninase production by *Wickerhamomyces subpelliculosus*.

Figure 4



Effect of different nitrogen sources on growth and L-methioninase production by *Wickerhamomyces subpelliculosus*.

pronounced increase of both growth and enzyme activity by increasing orange pulp concentration, and the optimum concentration was determined to be 15 g/50 ml medium. In this regard, Aggelopoulos *et al.* [24] mentioned that the increase of orange pulp concentration in a substrate mixture consisting of cheese whey, molasses, and potato pulp increased cell mass yields of *Kluyveromyces marxianus* and *Saccharomyces cerevisiae*. This increase in cell mass yield may be owing to the presence of orange pulp containing complex B vitamins that contribute to the transformation of pyruvate into acetyl-CoA, which enters the citric acid cycle to carry out metabolism and growth.

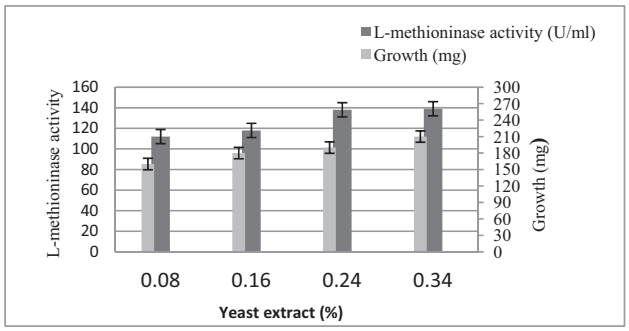
Effect of different nitrogen sources

Nitrogen source plays an important role in the synthesis of various cellular proteins including enzymes. Studies on the effect of different organic and inorganic nitrogen sources on L-methioninase production by *W. subpelliculosus* revealed that the enzyme production was markedly affected by the type of the nitrogen source used (Fig. 4). The supplementation of organic nitrogen sources to the growth medium enhances the production of L-methioninase. In this regard, yeast extract and peptone were found to be the best nitrogen sources for both yeast growth and L-methioninase production (90 U/ml and 78 U/ml respectively). On the contrary, the presence of inorganic nitrogen sources was not suitable for obtaining high enzyme levels. However, ammonium nitrate and ammonium phosphate gave moderate enzyme levels (51.0 U/ml). Regarding the effect of different concentrations of yeast extract (0.08, 0.16, 0.24, and 0.34%) on L-methioninase production, it is clear that the optimal yeast extract concentration was determined to be 0.34% (w/v) (Fig. 5). Peptone and yeast extract are complex ingredients containing several essential amino acids as well as many other growth-promoting factors, causing a profound increase in yeast biomass and enzyme production. The stimulatory effect of organic nitrogen sources like peptone and yeast extract on the production of other enzymes has been reported by many authors [35,36]. In addition, Arfi *et al.* [27] reported the use of peptone and yeast extract for the production of L-methioninase by *Geotrichum candidum*.

Effect of corn steep liquor as a nitrogen source on L-methioninase production

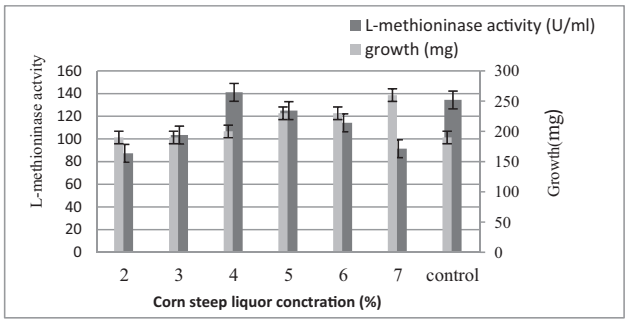
The high cost of yeast extract means that the industrial production of L-methioninase becomes quite expensive. It is desirable to find other more cost-effective and functionally comparable nitrogen sources such as CSL to develop more economic growth medium. Therefore, the effect of different concentrations of CSL on yeast growth and L-methioninase production was investigated in the presence of orange pulp as a carbon source. The production medium containing yeast extract served as a control. As shown in Fig. 6, the presence of CSL at a final concentration 4% (v/v) supported

Figure 5



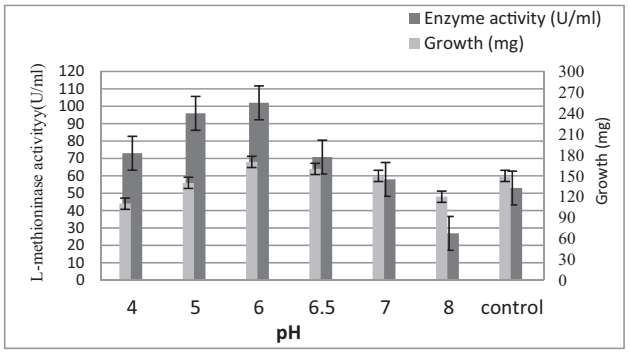
Effect of different yeast extract concentrations on growth and L-methioninase production by *Wickerhamomyces subpelliculosus.*

Figure 6



Effect of different corn steep liquor concentrations on growth and L-methioninase production by *Wickerhamomyces subpelliculosus.*

Figure 7



Effect of different pH values on growth and L-methioninase production by *Wickerhamomyces subpelliculosus.*

maximum enzyme production (141.12 U/ml), whereas the highest yeast growth was noticed at 7% (w/v). Moreover, the presence of CSL as a sole nitrogen source gave a higher enzyme level compared with that obtained with yeast extract (control). This finding may be owing to the fact that CSL, a by-product of the corn wet-milling industry, is rich in nutrients such as carbohydrates, amino acids, minerals, vitamins, and phosphates, so that it is considered as an excellent culture medium for enzyme production [37,38]. Similar results were reported by Xiong *et al.* [39], who used CSL as a supplement with wheat bran for the production of inulinase by *Kluyveromyces* S120. Based on the aforementioned results, CSL as an inexpensive nitrogen source could perform as yeast extract does in terms of enzyme production. A mixture of orange pulp and CSL was chosen as the most favorable agro-industrial by-products for L-methioninase production by *W. subpelliculosus*. It is difficult to compare the results obtained herein to other publications because the previous studies have been focused on the production of L-methioninase using the synthetic medium.

Effect of initial medium pH

The initial pH of the culture media was found to be critical for growth and enzyme production, as the metabolic activities of microorganisms are very sensitive to pH change. As shown in Fig. 7, L-methioninase production by *W. subpelliculosus* was favored by slightly acidic pH and showed the highest activity at pH 6.0 (102 U/ml). A similar pH value (5.5) was reported for L-methioninase production by *S. cerevisiae* and *Kluyveromyces lactis* (34 and 40, respectively) [40]. On the contrary, a reduction of both growth and enzyme production at higher pH values may be owing to the change in pH, which affects the cellular ionic equilibrium and metabolic enzymes, which are particularly sensitive to such variations [35,41].

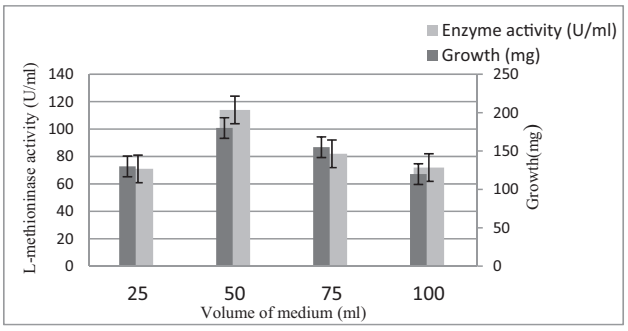
Effect of aeration level on L-methioninase production

It is important to supply enough oxygen to maintain the dissolved oxygen content in the medium for yeast cells at the optimal level [42]. L-methioninase production was greatly affected by the level of aeration, where increasing aeration level had a positive effect on yeast growth and enzyme production (Fig. 8). Results show that the air: medium ratio (4 : 1) corresponding to 50 ml medium/250 ml flask was the optimum ratio for both growth and enzyme production (200 mg and 114.24 U/ml, respectively). In this concern, Lin *et al.* [43] reported that the presence of an appropriate amount of dissolved oxygen made the yeast physiologically healthy and became productive.

Effect of metal salts

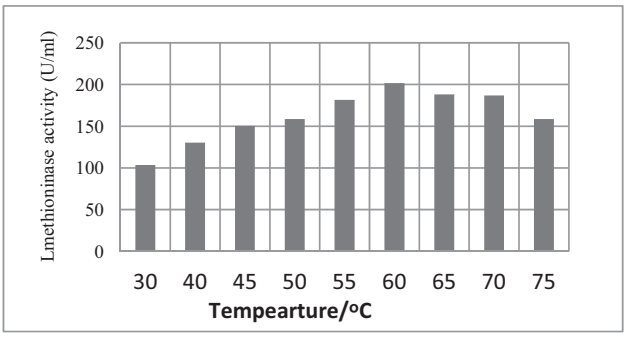
Metal ions affect the growth of yeast by influencing several metabolic activities and by acting as enzyme cofactors. A study on the effect of different metal salts (MgSO4, KCl, KH_2PO_4, and $CaCl_2$) on L-methioninase production indicated that some metal ions were stimulating, whereas others had no effect on enzyme production (Table 2). The high enzyme level

Figure 8



Effect of aeration level on growth and L-methioninase production by *Wickerhamomyces subpelliculosus*.

Figure 9



Effect of reaction temperature on L-methioninase activity.

Table 2 Effect of metal ions on growth and L-methioninase production by *Wickerhamomyces subpelliculosus*

Metal salts	Growth (mg)		Enzyme activity (U/ml)		Specific activity (U/mg protein)	
Concentration (%)	0.05	0.10	0.05	0.10	0.05	0.10
$MgSO_4$	231.5±2.12	240.5±0.70	140.23±0.66	143.24±3.17	34.39	33.477
KH_2PO_4	195±7.10	182.5±3.53	154.9±4.33	144±1.52	34.40	34.40
KCl	184±5.65	198.5±2.12	127.66±1.88	135.55±4.87	34.40	31.60
$CaCl_2$	283±4.24	267.5±3.53	160.95±2.89	159.2±5.37	34.39	27.43
Control	222.5±3.53		150.73±9.41		30	

Data is expressed as mean±SD of triplicates.

was obtained in the presence of $CaCl_2$ (160.95 U/ml) followed by KH_2PO_4 (154.90 U/ml) at a final concentration of 0.05% (w/v). Calcium chloride can control pH of the growth medium and has its own effect on yeast growth [44]. On the contrary, phosphate is considered as an important constituent of cellular biomolecules such as nucleic acids and co-enzymes and is known to play a regulatory role in the synthesis of primary and secondary metabolites in microorganisms [45]. In this finding, Sarlin and Philip [44] reported the presence of 0.15% calcium chloride and 0.3% potassium phosphate in the medium was the most favorable for yeast growth.According to the results mentioned before, we proved the ability of *W. subpelliculosus* (in terms of both yeast growth and L-methioninase production) to grow in an inexpensive medium containing orange pulp 30% (w/v), CSL 4% (v/v),$CaCl_2$ 0.05%, and KH_2PO_4 0.05% (w/v).

Physicochemical properties of partially purified L-methioninase

Optimum reaction temperature

Partial purification of crude L-methioninase from *W. subpelliculosus* was achieved by heat treatment. The highest specific activity (120.25 U/mg protein) was obtained at 70°C for 10 min, which resulted in 2.9 purification fold (data not shown). Regarding the effect of temperature on the enzyme activity (Fig. 9), it is evident that increasing the reaction temperature increased enzyme activity, reaching its optimum value at 60°C (200 U/ml). Moreover, the enzyme was highly active over temperature range 55–70°C. In this respect, the optimum temperature of *W. subpelliculosus* L-methioninase was higher than that reported for L-methioninase purified from *Candida tropicalis,* which was found to be 45°C [19,45].

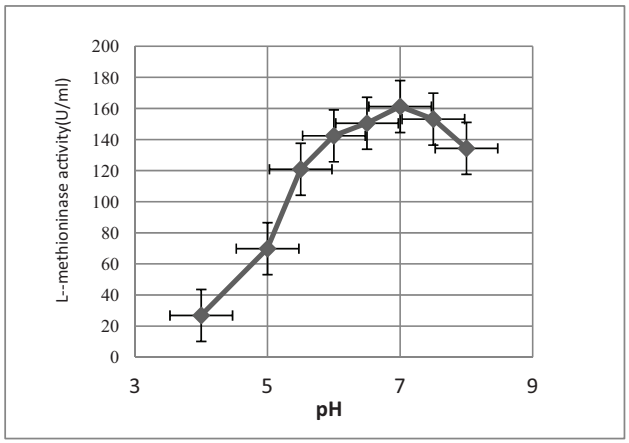
Optimum pH

The optimal pH for L-methioninase activity was determined by measuring the enzyme activity at different pH values using 50 mM sodium citrate buffer (pH 4.0–6.0) or 50 mM potassium phosphate buffer (6.0–8.0). As shown in Fig. 10, L-methioninase exhibited the highest activity at pH 7.0 (161.28 U/ml) and retained approximately 85% of its original activity at pH 8.0. Similar results were reported by El-Sayed [46] who mentioned that *Aspergillus flavipes* L-methioninase was optimally active in neutral to slightly alkaline pH value (7.8). In addition, the maximum activity of L-methioninase obtained from *G. candidum* and *S. cerevisiae* was observed at pH value 8.0 (34 and 47, respectively) [47].

Conclusion

This study points out for the first time that the utilization of a mixed formulation of orange pulp and CSL for developing an economic medium

Figure 10



Effect of different pH values on L-methioninase activity.

for the production of L-methioninase by *W. subpelliculosus* is possible. The developed medium formulation increased L-methioninase production (160.93 U/ml) by twofold compared with that obtained in Czapek–Dox's medium (73.92 U/ml). The significance of this investigation is attributed to the utilization of low-cost agro-industrial by-products for large-scale production of L-methioninase, which would get good economic returns and contribute to diminish the environmental pollution.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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