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Utilization of waste loquat (*Eriobotrya japonica* Lindl.) kernel extract for a new cheap substrate for fungal fermentations

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Abstract

In the present research, the usability of the extract prepared from waste loquat (Eriobotrya japonica Lindl.) kernels was investigated, as substrate in submerged cultures of moulds (Aspergillus niger and Rhizopus oryzae) and yeasts (Rhodotorula glutinis and Saccharomyces cerevisiae). Loquat kernel was found to be very rich in protein (22.5%) and total carbohydrate (71.2%) contents. The hydrolysate, which was generated by acid hydrolysis (2 N H_2SO_4) from loquat kernels, was neutralized, dried and converted to loquat kernel extract (LKE). In order to appraise the effectiveness of LKE on biomass production, it was compared with malt extract (ME), universally used for the cultivations for moulds and yeasts. ME resulted in slightly higher biomass yields than LKE; however, the test fungi S. cerevisiae grew comparatively well on LKE. The maximum productions of A .niger, R. oryzae, R. glutinis and S. cerevisiae biomasses on LKE were 19.53, 18.26, 8.8 and 3.9 g/L, respectively. LKE was also found to promote the mycelial pellet formation in test moulds. The usability of waste loquat kernels as a general fermentation substrate in submerged culture was demonstrated for the first time in the present study.

Key words: Waste material, Loquat kernel, Acid hydrolysis, Substrate, Fungal growth

Introduction

In the development of an optimized process for commercial production of microbial products, selecting a suitable low-cost nutrient medium is usually considered as a major aspect for improvement [1]. In the recent years, several reports have described the utilization of agricultural origin wastes as substrates in the culture mediums of microorganisms, for the production of commercially valuable products [2-6].

Loquat (*Eriobotrya japonica* Lindl.) is a subtropical evergreen fruit tree, native to the southeast of China, belonging to the Maloideae subfamily of the Rosaceae. Loquat is cultivated in Cyprus, Egypt, Greece, Israel, Italy, Spain, Tunisia and Turkey. It is also widely distributed in many European, Asian, and American countries [7,8]. According to the 2003 data, Turkey has some 288,000 loquat trees and these yield 13,000 tones of fruit [9]. The golden fruits are round or oval in shape and have a sweet taste. The fruits have various tissues, namely epidermis or epicarp, flesh or mesocarp (edible portion of the fruit), integument (very thin layer covering the seed), one to four seeds in each fruit and a hairy receptacle. In terms of weight, the seeds comprise about 20–30% of the weight of the whole fruit [10,11]. In traditional Japanese lore, loquat seed is called ‘‘good for health”. Village farmers soak the loquat seeds in alcoholic drinks. They believe that this type of drink is good for the health [12]. However, there is not enough information about the others employment fields of loquat seeds in the world. Especially in Turkey, the seeds are not utilized for any purpose after consumption of fruit flesh and currently discarded.

Loquat kernel is an agricultural origin waste that contains nutritional components needed for microbial activities. In spite of this, utilization of waste loquat kernel as substrate for

microorganisms has not been yet studied in detail. Usability of this kernel as substrate was investigated for only α-amylase and scleroglucan production [5,13]. Therefore, in this research we evaluated the usability of the extract obtained from waste loquat kernels as general fermentation substrate in submerged culture of moulds and yeasts.

Materials and methods

Microorganisms

While the microorganisms used in this study were determined, we paid attention to selection of ones that may be useful and attractive for the industrial purposes. The test moulds *Rhizopus oryzae* and *Aspergillus niger* were isolated from the campus soil of Ataturk University, Erzurum, Turkey. The yeast *Rhodotorula glutinis* was isolated from soil. Their isolation processes were performed on potato dextrose agar (PDA) by serial dilution of the samples, according to standard techniques [14]. The moulds were identified using mature cultures on PDA in order to ensure a good development of taxonomically relevant features, and following the identification keys [15,16]. The taxonomical idendification of the yeast *R. glutinis* was done by using the VITEX 2 compact device (Biomerux Company; Marcy, France). *Saccharomyces cerevisiae*, which is used for making bread, was chosen as the other test yeast. The cultures were maintained on PDA slants at 4°C and recultured bimonthly.

Preparation and chemical analysis of Loquat Kernel

Loquat fruit (1000 g) was purchased from a local market in Erzurum, Turkey. Firstly, the seeds were manually removed from fresh (edible parts) and others tissues. The seed testa or seed skin was separated from kernel, and loquat kernel was recovered. Kernels were cleaned one time with deionized water to remove the undesirable materials, and then external moisture was wiped out with a dry cloth. Moisture, crude protein, crude fat and ash contents of kernel were determined according to AOAC methods: 925.10, 955.04, 920.39 and 923.03, respectively [17]. Nitrogen content was measured using a micro-kjeldahl apparatus (Labconco; Labconco Corp., Kansas City, MO, USA) and crude protein was estimated by multiplying nitrogen content by 6.25. Crude fat was determined by ether extraction in a soxhlet apparatus. Ash was determined by combusting dry sample in a muffle furnace **(**Thermolyne 62700; Barnstead/Thermolyne Corp., Dubuque**,** Iowa**,** USA**).** Total carbohydrate content was calculated by difference [Carbohydrate = 100% - (% protein + % fat + % ash)].

Preparation of loquat kernel extract (LKE)

The loquat kernels dried at 105°C were ground with a grinder (Wiley-mill; Arthur, USA). This material (particle size 1 mm) was termed as loquat kernel flour (LKF). Firstly, 100 g of LKF was mixed with 500 mL of H_2SO_4 solution in an autoclavable bottle and volume was made up to 1 L with deionized water. After the bottle was introduced into an autoclave (HVE-50; Hirayama corp., Tokyo, Japan), hydrolysis process of LKF was started. During the hydrolysis experiments, the different concentrations (0.5, 1, 1.5, 2 and 2.5 N) of H_2SO_4 solution and hydrolysis times (15, 30, 45, 60, 75 and 90 min) were tested. All hydrolysis experiments in autoclave were carried out at 121°C under pressure of 1.2 atm. After hydrolysis, the solution was cooled to room temperature, and pH value was adjusted to 7.0 with 10 N NaOH. The liquid and solid fractions of each solution were separated from each other by filtration using Whatman No. 1 filter paper. Then, the solid fraction was washed twice with hot deionized water, and washing water was combined with the liquid fraction. Finally, the total volume of each liquid fraction was completed to 2 L with deionized water and its total sugar content was determined by the Nelson's reducing sugar test with glucose as a standard [18]. Absorbance was recorded at 500 nm using a spectrophotometer **(**UV-160A**;** Shimadzu Corp., Kyota, Japan**).** Deionized water was used as control. Among all the liquid

fractions, the fraction having the highest total sugar content was chosen and termed as loquat kernel hydrolysate (LKH). It was dried at 105°C until constant weight and then, it was powdered. The dried and powdered soluble material was termed loquat kernel extract (LKE) and used as main carbon source in the growth media.

Preparation of inoculum

The mould isolates were initially grown at 30°C on PDA slants. At the end of 6-days incubation period, ten milliliters of sterile saline water (0.9% NaCl) were added to culture slant of each isolate, and these slants were vortexed. The final concentration of the spore suspension of each test mould was adjusted to 10^6 spores/ml, with sterile saline water. To prepare the yeast starter, 250-ml Erlenmeyer flasks containing 100 ml of patato dextrose broth (PDB) was inoculated with one loopful of a 24 h-old culture of yeast grown on PDA and then incubated at 30^0C and 200 rpm for 48 h on a rotary shaker (Gallenkamp; USA). After growth, the yeast cells were collected by centrifugation at 5000 g for 15 min and resuspended in sterile saline water. The cell final concentration for the test yeasts was adjusted to10^6 cells/ml.

Biomass production studies on loquat kernel extract medium

The biomass production studies in liquid culture for the test microorganism were aerobically carried out in loquat kernel extract medium (LKEM) and malt extract medium (MEM). These mediums were prepared by solving peptone (5 g/L) and extract in distilled water. The pH value of each medium was adjusted to 5.5. The production studies of biomasses were carried out in 250-mL flasks containing 100 ml of one of the two extract media. For this, each flask was autoclaved at 121°C for 15 min, cooled at the room temperature, inoculated with 1.0 ml of the cell suspension of yeast or the spore suspension of the mould under sterile conditions. The flasks were incubated at 30°C and 200 rpm on a rotary shaker. During the experiments, we firstly investigated the effect of various concentrations (10-80 g/L) of LKE on the mycelium growth or cell growth of the test moulds or yeasts. To determine the optimum concentration of LKE, the yeasts were cultivated in LKEM for 2 days and the moulds for 3 days. All the experiments were performed at least in duplicate and the average values were used in calculations.

Analysis of fermentation parameters

To measure the residual sugar concentration in culture media of the fungi, 1 mL sample taken from each culture broth after every 24 h was centrifuged at 5000 g for 15 min, and the obtained clear supernatant was suitably diluted and then analyzed with the DNS method as described by [19]. To determine the dry cell weights of yeasts, the yeast cells obtained at the end of the centrifugation process were washed three times with distilled water and then dried at 105°C until constant weight. To determine the biomass concentrations of the moulds, the mycelia of each mould was separated from the culture broth under vacuum, washed three times with distilled water and then dried at 105°C until constant weight.

Results and discussions

Chemical analysis of loquat kernel

Loquat fruit tissues were separated as described in the Materials and methods section. At the end of this, it was determined that 1000 g of fruits contained 250 g seeds (by wet weight). Kernels accounted for approximately 94% of seed and 22.5% of whole fruit (by wet weight). That is to say, 1000 g of fruits contained 225 g kernels.

The moisture content of kernel was on average 9.8%. Crude protein, crude fat, crude ash and total carbohydrate contents of kernel were found to be approximately 22.5, 3.4, 2.9 and 71.2% on dry basis, respectively. These results meant that loquat kernel had high protein and total carbohydrate contents but low ash and fat contents. Since protein is necessary for microbial growth, the supplementation with vegetable protein will support large scale cultivation of

mould for the production of valuable microfungal biomass and probably for enzyme and antibiotic production [4]. The high protein content of loquat kernels is an indication that the waste could serve as a possible alternative substrate for cultivation of microorganisms and support the production of valuable microbial products. The total carbohydrate content of loquat kernel is high and could serve as the main carbon source for microbial growth. Ash is a reflection of the amount of mineral elements in the samples and therefore serves as the main source of mineral elements needed for microbial activities. Although the ash content of loquat kernel was low, salts of mineral elements may be incorporated into the media during formulations. In this context, the addition of peptone into the culture medium can provide these elements to the test fungi. Besides, the usage of NaOH for the neutralization of the hydrolysate increases the concentration of Na element.

Preparation of LKE

Kernels dried at 105^0C were powdered (particle size 1 mm) and loquat kernel flour (LKF) was thus obtained. To prepare neutralized loquat kernel extract (LKE), 100 g/L of LKF was used in the hydrolysis experiments. Following hydrolysis, the solutions formed in flasks were neutralized. After solid-liquid separation, the total sugar content of liquid fraction was determined. Fig. 1 clearly indicates that the liquid fraction obtained with a 2 N H_2SO_4 solution for 60 min contained the highest total sugar content (62.8 g/L) and this fraction was chosen as LKH and used as main carbon source in the following experiments. At the end of hydrolysis process, 28 g non-hydrolyzed solid fraction was obtained. This meant that 72 g/L soluble material was obtained from 100 g/L of LKF with acid hydrolysis. On the other hand, 26 g NaOH was used for the neutralization of this soluble material. In this way, 98 g/L total soluble material (LKH) was obtained. This demonstrated that the acid hydrolysate needed large amounts of NaOH for neutralization. This result is consistent with the fact that although acid hydrolysis allows high yields, this process results in high ash content in the final products as the neutralization step cannot be avoided [20,21]. After LKH was dried and powdered, 98 g of LKE containing 62.8 g of total soluble sugar was obtained. On the other hand, it was observed that the total sugar content of the hydrolysate obtained with 60-min hydrolysis time was higher than those of the hydrolysates obtained with 75 and 90-min hydrolysis times. The similar trend was also noted when the hydrolysis process of LKF was carried out with 2.5 N solution of H_2SO_4: the total sugar content of the obtained hydrolysates increased with the increase of hydrolysis time from 15 to 45 min but decreased with a further increase in hydrolysis time from 45 to 90 min. This must be due to the degradation of monosaccharides in the hydrolysate.

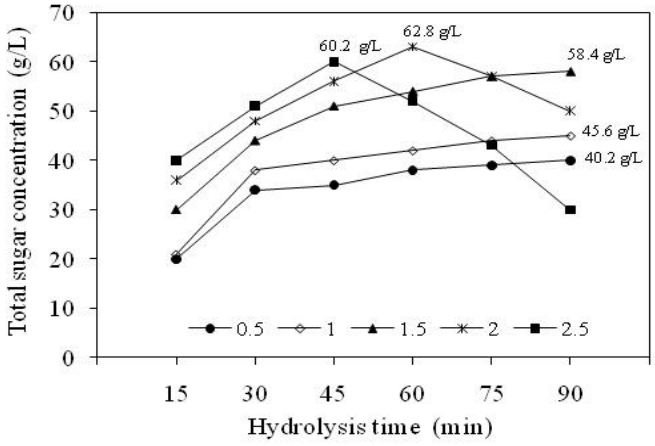

Figure 1. Time profile of total sugar contents in the hydrolysates prepared from 100 g loquat kernel flour (LKF) at the different H_2SO_4 concentrations. Hydrolysis conditions in autoclave: 121°C and 1.2 atm. For each hydrolysis condition, maximal values are annotated.

Determination of the optimal concentration of LKE

To evaluate the usability of LKE as a substrate, four fungi that are commonly used for the production of valuable microbial products were cultivated in LKEM containing LKE and peptone. We investigated the effect of LKE in various concentrations (10–80 g/L) on biomass production and the obtained results are shown in fig. 2. The maximum values for both yeasts and moulds biomasses were reached when 60 g/L of LKE was used in the culture media. They were 16.1, 13.2, 7.6 and 3.18 g/L for *A.niger, R.oryzae, R.glutinis* and *S. cerevisiae*, respectively. Above this optimal concentration of LKE, higher applications had an inhibitory effect. This inhibitory effect may be due to the presence of high salt concentration and some toxic materials in LKE. Hence, the optimum concentration of LKE for the subsequent biomass production studies was selected as 60 g/L.

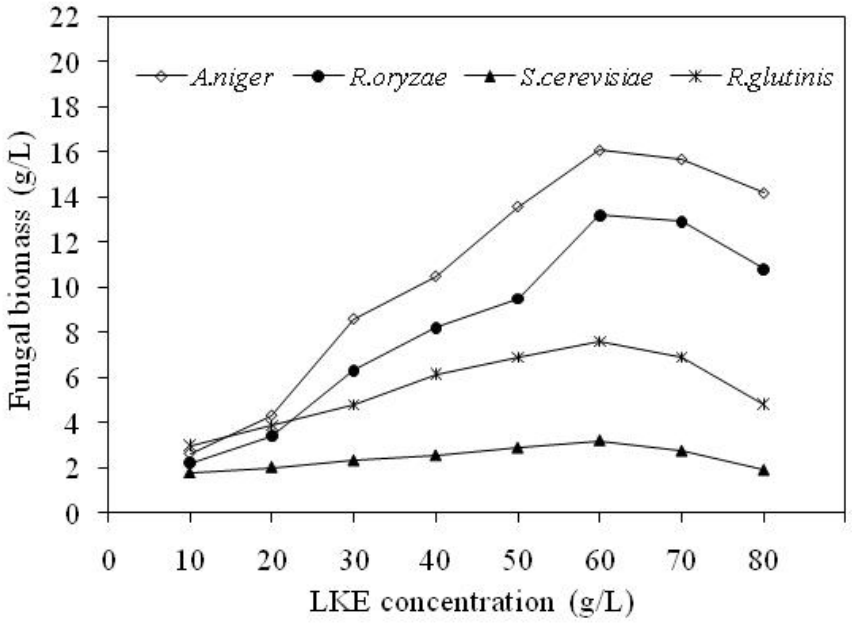

Figure 2. Biomass production by yeasts (for 2 days) and moulds (for 3 days) at the different loquat kernel extract (LKE) concentrations. Fermentation parameters: Peptone = 5 g/L, Cultivation temperature=30˚C, pH = 5.5 and Shaking speed=200 rpm.

Determination of yeast and mold growth on LKEM and MEM

In order to appraise the effectiveness of LKE on biomass production, it was compared with ME universally used for cultivation of yeasts and moulds. Concentration of each extract added in the culture medium was adjusted to 60 g/L, which was determined as optimal concentration of LKE for all the test fungi. The results demonstrated that the sugar content of LKEM was completely exhausted at the end of 5th day of cultivation (Fig. 3A), when the mycelial biomass yields of *A. niger* and *R. oryzae* reached 19.53 and 18.26 g/L (Fig. 3B), respectively. After 5th day of cultivation, there was a decrease in the total biomass amounts of both moulds. This situation may be attributed to fungal cell lysis, which appears with the depletion of sugar content in the culture medium. In comparison with LKEM, MEM caused longer fermentation time for both moulds. Therefore, the maximum biomass values for moulds were reached at the end of the 6th day, with the complete depletion of sugar content in MEM (Fig. 4A and B). These values (21.3 and 20.12 g/L for *A. niger* and *R. oryzae,* respectively*)* were slightly higher than those obtained with LKEM.

On the other hand, no significant difference was observed between the fermentation courses of cultures grown on LKEM and MEM. The sugar contents of LKEM and MEM were exhausted rapidly from the beginning of the 2nd day up to the end of 3th day and slowly from the beginning of the 4th day up to the end of the fermentation period. According to the results obtained from the comparison of LKE with ME, LKE was a favorable substrate for the

cultivations of *A. niger and R. oryzae,* since it resulted in biomass yields near to those obtained with ME.

Fungal growth in pellet form is favorable for the fungal fermentations since it not only makes repeated-batch fungal fermentation possible but also significantly improves the culture rheology which results in better mass and oxygen transfer into the biomass and lower energy consumption for aeration and agitation [22]. Obtaining uniform pellets of a desired size is not easy, since many factors influence pellet formation: inoculum size, type and age, genetic factors, ability to produce bioflocculants, medium composition, shear forces, pressure, temperature, medium viscosity and oxygen concentration [23]. The experiments displayed that the mycelial pellet formation in the test moulds was strongly associated with extract kind used in the culture medium. This finding is in accordance with the fact that the mycelia pellet formation in filamentous fungi *A. niger* and *R. oryzae* is influenced by the nutritional composition of culture medium [24-26]. Large uniform pellets with diameter of 2.5 mm for *A. niger* were formed in MEM, while small uniform pellets with diameter of 2 mm were formed in LKEM. *R. oryzae* grew in a small uniform pellet form with diameter of 1.5 mm when cultivated in LKEM, while it grew in a clump form when cultivated in MEM. These results concluded that LKE was a better nutritional substrate than ME for fungal growth in small uniform pellet form. This finding may make LKE an indispensable nutritional substrate in industrial fermentation studies, because the small uniform pellet form is usually preferred in fungal fermentation studies. Large pellets may limit internal mass transfer, resulting in a low reaction rate and, consequently, a decrease in the production rate [27].

Several studies showed that *A. niger* and *R. oryzae* strains were able to produce valuable industrial products when cultivated on agricultural origin substrates [3, 28-32]. Considering the positive effects of LKE on biomass production and mycelium morphology, it is possible to say that LKE prepared from waste loquat kernels may be utilized as substrate in the culture media of *A. niger* and *R. oryzae,* for the productions of valuable industrial products, along with the utilization of other agricultural origin substrates.

The maximum biomass productions for the test yeasts on MEM were reached at the end of the 7th day, when the sugar content in the culture medium was completely exhausted (Fig. 4A and B)**.** These values were 10.23 and 11.7 g/L for *R. glutinis* and *S. cerevisiae*, respectively. The experiments also displayed that the cell growth in both yeasts was faster within the first 3 days of cultivation (especially the fastest within the first day of cultivation) and slow after 3th day. The performed experiments demonstrated that the growth performances of *R. glutinis* and *S. cerevisiae* on LKEM were very different from each other. *R.glutinis* cells showed good growth performance on LKEM. Therefore, the cell biomass weight of *R.glutinis* reached to maximum (8.8 g/L) at the end of 6th day, with the complete depletion of sugar content in the culture medium (Fig. 3A and B). In contrast to *R.glutinis* cells, *S. cerevisiae* cells showed relatively poor growth performance in LKEM. Thus, less biomass (3.9 g/L) for this yeast was attained at the end of 6th day of cultivation. Besides, relatively high amount (13.5 g/L) of residue sugar was recorded in LKEM at the end of 6th day of cultivation due to the poor growth performance of *S. cerevisiae* cells. From fig. 3A and B, it can be also concluded that the fermentation process in LKEM for *R. glutinis* cells was similar to that in MEM. Namely, the fermentation was the fastest within the first day of cultivation and slowly progressed after the 3th day. These results meant that LKE was a good substrate for the cell growth in *R. glutinis* but unfavorable substrate for the cell growth in *S. cerevisiae*. Therefore, LKE may be an alternative substrate in the industrial fermentation studies, particularly where the carotenoid production by *R. glutinis* is carried out. This assumption may be also supported by some studies, which show that agricultural origin substrates for carotenoid production by *R. glutinis* can be effectively used [1,33,34].

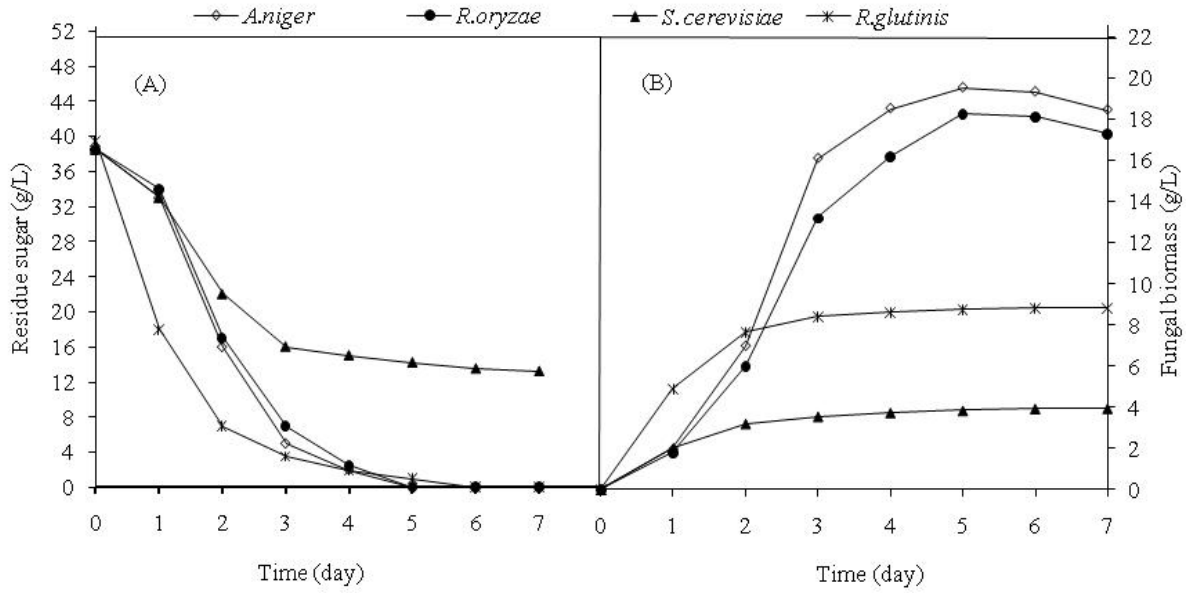

Figure 3. Time profiles of residue sugar (A) and fungal biomass (B) in the cultures of the test fungi cultivated in LKEM. Fermentation parameters: Loquat kernel extract (LKE) = 60 g/L, Peptone = 5 g/L, Temperature = 30˚C, pH = 5.5 and Shaking speed = 200 rpm.

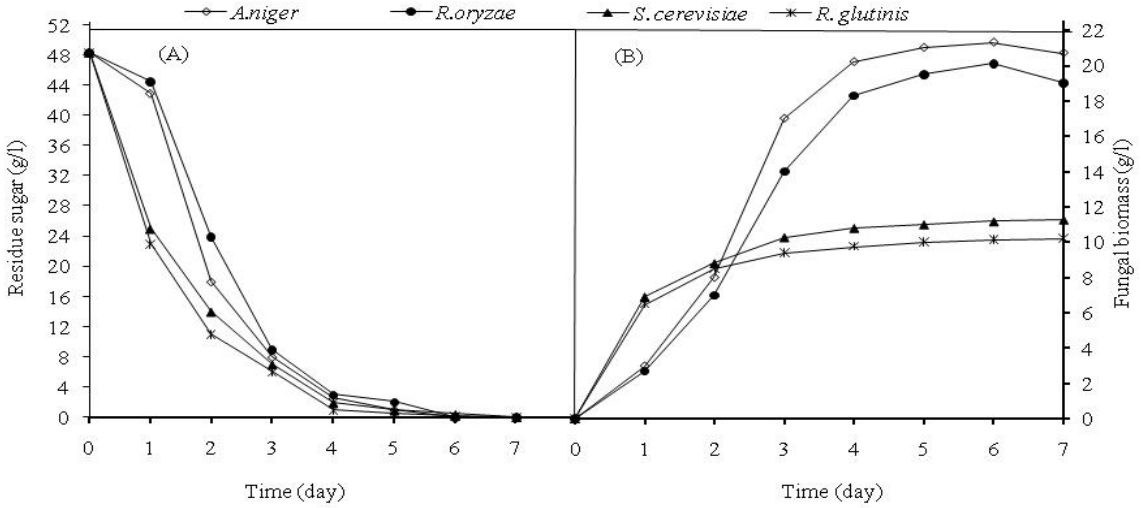

Figure 4. Time profiles of residue sugar (A) and fungal biomass (B) in the cultures of the test fungi cultivated in MEM. Fermentation parameters: Malt extract (ME) = 60 g/L, Peptone = 5 g/L, Cultivation temperature = 30˚C, pH =5.5 and Shaking speed = 200 rpm.

It is generally accepted that carbon sources are more associated to cell growth and nitrogen sources to metabolite production. Accordingly, shorter fermentation time and lower biomass yield in LKEM might be due to the fact that the total sugar content of LKE was lower than that of ME, as 60 g ME contained 48.4 g total sugar, whereas 60 g LKE contained 38.5 g total sugar. In essence, the hydrolysis process of 100 g/L LKF resulted in high sugar content (62.8 g/L); however, the high ash content occurring after neutralization decreased the percentage of sugar concentration in the hydrolysate. At this point, the different hydrolysis processes that result in lower ash content may be tested in order to increase the percentage of carbon source, which is a critical factor affecting the fermentative process to produce microbial biomass. Although LKE contained lower total sugar content than ME, it was very interesting that LKE caused biomass yields near to those obtained with ME. This must be due to the chemical composition of LKE.

Many investigators have reported that some toxic compounds such as furans, phenolics and acetic acid occur in the hydrolysates of lignocellulosic materials, and these compounds negatively affect the growth performances of microorganisms [2,35,36]. Therefore, poor growth and slow sugar consumption capacities of especially *S. cerevisiae* on LKEM may be the result of the presence in LKE of these toxic compounds. In this context, some detoxification methods, such as overliming with calcium hydroxide and activated charcoal may be used to remove toxic compounds from LKE. On the other hand, many investigators reported that the nutritional compositions of hydrolysates or extracts could be improved when they are enriched with some additional carbon, vitamin and mineral sources[1,2,31]. Therefore, the modification of LKE with these additional substances may make this extract more suitable for industrial fermentation studies. However, further studies need to prove these assumptions.

With the rapid industrial development and population increase, large amounts of wastes containing carbohydrate, protein and other basic nutrients have been produced and discharged into environment. Accordingly, these wastes have led to the environmental problems. On the other hand, the cheap and useful substrate requirement for the industrial fermentation studies has continuously increased. Loquat kernels are currently discarded as waste material without being utilized. In the present study, 1000 g of loquat fruits was found to contain 225 g kernel (by dry weight). Taking into account of this rate, 225 kg of loquat kernels are generated as waste from 1000 kg of loquat fruits. The present study indicated that LKE was a good substrate for the cultivations of the test fungi *A.niger*, *R.oryzae* and *R.glutinis* but an unfavorable substrate for the cultivation of the test fungus *S. cerevisiae*. It was also found that LKE resulted in small uniformed pellets, which is usually preferred in fungal fermentation studies. In this context, the utilization of waste loquat kernels as a source of nutritional components needed for microbial activities in the production studies of valuable industrial compounds, not only provides the cheap substrates to industrial fermentation studies but also helps the solution of environmental problems by reducing the amount of wastes, which may cause serious ecological hazards. Therefore, this waste management process is both economical and environmentalist. On the other hand, the suitability of LKE as substrate in bacterial growth media is currently under investigation in our laboratory.

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