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Bioremediation of phenolic compounds from fruits juices by immobilized laccase

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Phenolic compounds in orange juice was removal by using immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads. The yield and efficiency of immobilization were 89 and 93%, respectively. The optimum pH of activity was 5 and its loss 49.2 and 69.8% from initial activity at pH 3 and 7, respectively, it is stable at pH range 3-6 and lost 42% from initial activity at pH 7 for 15 min. The optimum temperature was 50°C and enzyme loss 44.1 and 64.4% from initial activity at 30 and 70°C, respectively, it is stable at 60°C for 15 min and lost 47.4 and 61.6% from initial activity at 65 and 70°C, respectively. The enzyme retained 100 and 78.29% from initial activity after storage for 24 and 30 day at 4°C, respectively, and retained all activity for 21 continue usage; whilst it retained 80.47% of its original activity after 30 continue usage. The treatment of orange juice by immobilized laccase lead to increase in transmittance to be 124.6% and color was reduced to be 33.7%. The estimation of the optimal time for removal of phenolic compounds from orange juice by immobilized laccase showed that the treated for 10, 20, 30, 40, 50 and 60 min lead to removed 18.37, 52.79, 89.16, 90.08, 91.02 and 91.06% of phenol compounds respectively. The stability of orange juice treated with immobilized laccase after heating, freezing and thawing was more than non-treated juice.

Keywords: Immobilized enzymes, laccase, calcium alginate beads, bentonite clay, bioremediation, removal of phenol compounds, orange juice clarification, enzymes applications.

INTRODUCTION

The consumption of fruit juices constantly increases in the world due to the beneficial effects of health and wellness (Alsoufi, 2021). Polyphenol compounds in fresh fruit juices are an important natural source of antioxidants as a benefit for consumers health (Kahraman et al.2017). However, these compounds are also causing changes in color, aroma and flavour, affecting ultimate juice shelf-life and consumer acceptability (Pezzella et al.2015). In order to decrease of this unacceptable phenomenon from producers and consumers of fruit juices, producers usually use physical and chemical clarification processes through filtration and adsorbents to remove haze and sediments or improvement color, aroma and flavor. The main problem of these type of processes is that produced fresh fruits juices are not constantly stable due it tends to produce clear haze and both type of browning reaction, caused by reactive of phenolic compounds residue. So, these compounds must be removal from fresh juices to save its quality and freshener (Agcam et al.2014; Lettera et al.2016).

Laccase, (EC 1.10.3.2) blue oxidases, copper enzyme, belong to the oxidoreductase enzymes groups. It is oxidation of a wide type of phenols (mono, di, poly, amino and methoxy), ascorbate, 4-hydroxybenzoic acid, aromatic amines, aniline and [2, 2'-azino-bis- (3-ethyl benzothiazoline-6-sulphinic acid)] (ABTS), The catalytic mechanism of the laccase depend on an electron donation from cu in active site to the substrate to reduction of oxygen to water (Fernández-Fernández et al.2012).

This enzyme is generally found in plants, insects, bacteria and fungi (Al-Soufi, 2016). It can be used in wide of biotechnological applications due to their ability to oxidize and degrade phenolic compounds, thus, there are use in industrial process such as in food technology, wastewater treatment, dye decolorization, textile, biosensors, cosmetics, biofuel cells, paper and pulp products, delignification, soil bioremediation, juice clarification and others (Narnoliya et al.2019; Alsoufi, 2021; Aziz 2021).

In recent years, research has been intensified in this field to detection efficient and economical methods to improve properties of fresh juice and reduce polyphenol compounds through its polymerization and subsequent ease of removal by use of immobilized laccases, (Al-Soufi, 2016c), The immobilization will be improving enzyme characteristics by increase in stability of pH and temperature, easily separated from the reaction solution and reuse ability for many times (Al-Soufi, 2016a, Alsoufi, 2019; Alsoufi and Aziz, 2019; Alsoufi and Aziz, 2020).

Generally, studies in this field have been carried out by different an insoluble support such as magnetic-nanoparticles (Narnoliya et al.2019; Wang et al.2020), magnetic chitosan–clay composite (Aydemir and Güler, 2015), Monoaminoethyl-N-aminoethyl agarose (Brugnari et al.2018), Bentonite (Alsoufi, 2018). So, this study aimed to encapsulation immobilized laccase within calcium alginate-bentonite beads and study of its application characteristics for bioremediation of phenolic compounds from fruits juices.

MATERIALS AND METHODS

Laccase

Laccase (EC 1.10.3.2) from *Agaricus bisporus* powder, deep brown, ≥4 U/mg, from SIGMA was used in this study.

Estimation of protein

The amount of protein (U/mg) was determined through a method of Bradford (1976) using bovine serum albumin as a standard protein.

Activation of clay (bentonite)

The activation of clay was carried out in the following method of Alsoufi (2021) occur by add clay to [10% 3-APTES solution in acetone (v/v)] and stirring for 1 min at 25°C, then filtered, washed with acetone and dried in oven at 80°C, then adding to 10% aqueous solution of glutaraldehyde (v/v) with stirring for 1 min at 25°C, then filtered, washed, dried at 25°C and stored in 20 mM citrate buffer pH 5 at 4°C until use to immobilization.

Encapsulation of immobilized laccase

The activated bentonite mixing to same amount of laccase (10 mg/mL) in 20 mM citrate buffer solution pH 5 and stirring for 1 min at 4°C, after that centrifuge at 5000 rpm for 30 min at 4°C, then, wash the clay for three times with the same buffer solution to removal all the free laccase in supernatant (Alsoufi, 2018), then, 2.5 gm of alginate powder was mixed with 2.5 gm of bentonite-laccase and added to the 100 mL of the same buffer and stirred for 4 h, at the end of stirrer time, it has been added 2.5 mL of glycerol to the alginate-bentonite-laccase solution. The mixture was stirred and dropped 0.2 M $CaCl_2$ solution for 3 h. After of hardening, gel beads were collected and washed with 20 mM citrate buffer solution pH 5 for three times and stored in the same buffer at 4°C until use (Al-Soufi, 2016c).

Laccase assay

The activity of immobilized laccase was estimated by using spectrophotometer at ʎmax = 420 nm (ε=36,000$M^{-1}cm^{-1}$) with 5 mM of ABTS as a substrate in 100 mM of sodium acetate buffer (pH 4.0) at 25°C by measuring the oxidation increase of ABTS during reaction time according to Alsoufi (2018). One unit of enzyme activity was defined as the amount of laccase required to oxidize 1 µmole of (ABTS) per 1 min at 25°C (Mi and Park, 2008).

Yield and Efficiency of the immobilization method

Immobilization yield of laccase and Efficiency were estimated with the following equation by Alsoufi and Aziz (2020); Yin et al. (2021):

$$\text{Immobilization yield (\%)} = \frac{\text{Activity } (\frac{\text{U}}{\text{mL}}) \text{ of immobilized laccase}}{\text{Activity } (\frac{\text{U}}{\text{mL}}) \text{ of initial free laccase}} \times 100$$

$$\text{Immobilization efficiency (\%)} = \frac{\text{Specific activity } (\frac{\text{U}}{\text{mg}}) \text{of immobilized laccase}}{\text{Specific activity } (\frac{\text{U}}{\text{mg}}) \text{ of initial free laccase}} \times 100$$

pH profile

The optimum of pH activity for enzyme was determining at pH range 3-8 by using 50mM of sodium-acetate and Tris-HCl to prepare buffer solution pH 3-6 and 6.5-8, respectively, with (ABTS) as the substrate; the optimum pH for stability was determining after incubation of enzyme with working solution buffer for 15 min (Alsoufi, 2021).

Temperature profile

The optimum temperature for activity was determining at 30-70°C using optimum pH of activity and (ABTS); while optimum temperature for stability was determining after incubation of enzyme at 30-70°C for 15 min (Alsoufi and Aziz, 2020).

Storage and reuse

The effect of storage on immobilized laccase was estimated through stored for 60 day at 4°C, while the effect of reuse was followed up to 40 time of use according a method of Alsoufi (2019).

Application

Juice preparation

Orange (*Citrus sinensis*) juice was prepared through a method of Alsoufi (2021) by washed, dryad, peeled and juice was extracted, then centrifuge at 3000 rpm for 10 min at 4°C to remove all precipitate and getting clear juice.

Determination of phenolic compounds

The extraction of phenolic compounds was carried out by mix juice with methanol (1:1) for 30 min at-18°C, then centrifugation for 30 min at 5000 rpm, the supernatant was used to determined total phenolic compounds content by using Folin-Ciocalteu assay and [gallic acid (3,4,5-trihydroxybenzoic acid) (10-100 mg/mL)] as calibration curve. The absorbance all samples were measured at 725 nm (Alsoufi and Aziz, 2020).

Bioremediation of phenolic compounds

The bioremediation of phenolic compounds (%) was determined by the method of Al-Soufi (2018) by add 2.5 gm of immobilized laccase (10 mg/mL) (20 U/mg) to the 1

L of orange juice and stirring for 30 min at 40°C and 50 rpm.

Transmittance

The transmittance of orange juice was measured at 650 nm with de ionized water as a blank (Deng et al.2019).

Color value

The color value was measured at 430 nm with de ionized water as a blank after the mixture equal volume of orange juice with of ethanol for 30 min (Deng et al.2019).

Estimation of the optimal time

The effect of time was studied according to the method described by Alsoufi (2018) by an add 2.5 g (10 mg/mL) (20 U/mg) to 1 L of orange juice and stirring for 0-60 min at 40°C and 50 rpm.

Stability of Orange juice

The stability of treated and non treated juice was measured by heated at 70°C for 30 min and froze at -18°C for 24 h, respectively. The light transmittance of orange juice was compared after treatment to determine the stability (Wang et al.2020).

RESULTS AND DISCUSSION

Yield and efficiency of Immobilization

The yield and efficiency of immobilization Laccase by calcium alginate-bentonite beads was 89 and 93%, respectively.

The estimation of yield and efficiency for immobilized enzyme represented the main step of immobilization process; which need to use an inert binding material such as polymers and inorganic materials that have a high strength, stability, sensible prices, keep of activity, bind the highest amount of enzyme and reusable. (Alsoufi, 2018), so, all methods of immobilization aim to improvement stability of pH and temperature, storage and reusability (Alsoufi and Aziz, 2020).

In this subject, Yin et al. (2021) found that the yield and efficiency of laccase Immobilization on magnetic nanoparticles were 84 and 91%, respectively. Narnoliya et al. (2019) refer that the immobilization yield was about 50% for enzyme on iron magnetic-nanoparticles. Alsoufi, (2018) found that observe that yield of immobilization for laccase on bentonite by glutaraldehyde was 91%. Brugnari et al. (2018) observe that the immobilization yield was 100% for laccase on MANAE-agarose. Mureşeanu et al.(2016) refer that the efficiency of laccase immobilization on hexagonal mesoporous silica (HMS-NH_2cov) was 81.86. Aydemir and Güler (2015) explain that immobilization yield is 75% using magnetic chitosan-clay beads. Patel et al. (2014) obtain of 75.8 and 92.9% yield and efficiency, respectively, of laccase on SiO_2 nanoparticles.

pH activity and stability

The optimum pH of laccase was 5 and enzyme loss 49.2 and 69.8% from initial activity at pH 3 and 7, respectively, (Figure 1), it is stable at pH range 3-6 and lost 42% from initial activity at pH 7 (Figure 2)

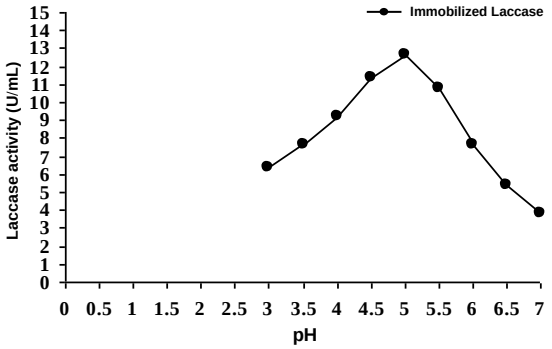

Figure 1: Optimum pH activity of immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads.

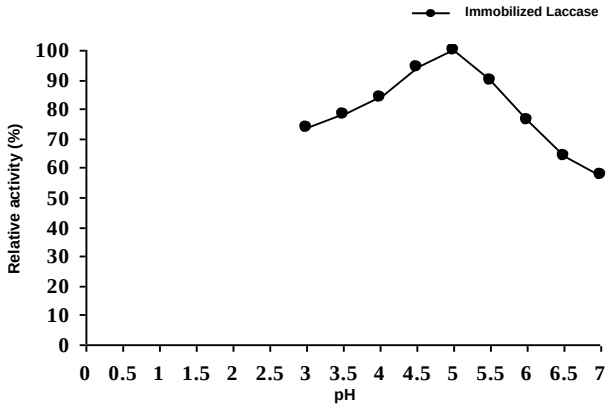

Figure 2: Optimum pH stability of immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads.

The pH of activity and stability of enzyme represent the main parameter for the success of immobilization process, the change of pH may lead to disability using it applications of it, therefore ending the feasibility of immobilization (Al-Soufi, 2015; Alsoufi and Aziz, 2020).

Many studies refer to this fact, the pH profile of immobilized enzyme from *Trametes versicolor* on magnetite nanoparticles of Fe_3O_4 was 4.0 (Wang et al.2020), and it was 5.5 for enzyme from *Bacillus atrophaeus* on magnetic particles of iron oxide (Fe3O4) (Narnoliya et al.2019), The immobilized laccase *Myceliophthora thermophila* on epoxy-functionalized silica exhibit maximal activity at acidic pH 3.0-5.0. (Mohammadi et al.2018), while The maximal pH activity was 5.0 for enzyme from *Pleurotus ostreatus* on MANAE-agarose (Brugnari et al.2018) and laccase from *T. versicolor* on magnetic chitosan-clay (Aydemir and Güler, 2015).

The improvement of immobilized laccase stability in acidic pH probably due to the covalent multipoint

attachment that increase of stability, as well as, the microenvironment of laccase on the bentonite could have buffering influence which lead to improve stability of enzyme at this condition (Yin et al.2021).

Temperature activity and stability

The optimum profile of laccase was 50°C and enzyme loss 44.1 and 64.4% from original activity at 30 and 70°C, respectively, (Figure 3), it is stable at 60°C for 15min and lost 47.4 and 61.6% from initial activity at 65 and 70°C, respectively, (Figure 4).

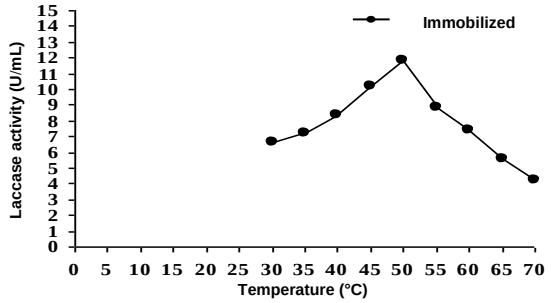

Figure 3: Optimum temperature activity of immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads.

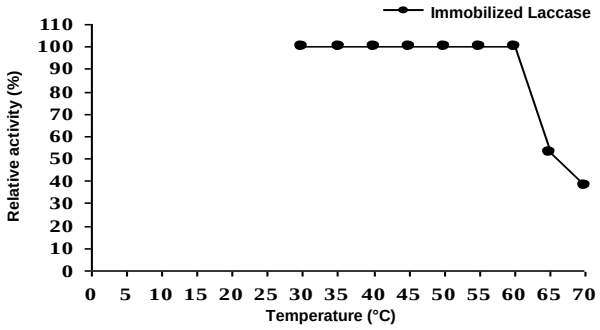

Figure 4: Optimum temperature stability of immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads.

At the high temperature, the thermal stability of laccase lead to the covalent bonds between amino and carboxyl groups of enzyme and clay, respectively, which will increase the hardness of enzyme structure and provided high protect of laccase molecule from unsuitable changes at high temperature which lead to exposed folds of the enzyme molecule and active site to reaction medium and denaturation of enzyme due to the Increase of temperature. (Alsoufi and Aziz, 2020; Yin et al.2021) therefore, the optimal temperature of enzyme from *T. versicolor* on magnetite nanoparticles of Fe_3O_4 was 50°C (Wang et al.2020), on magnetic chitosan-clay was 40°C (Aydemir and Güler 2015), and on SiO_2 nanoparticles was 45°C Patel et al.(2014), while it was 55°C for enzyme from *P. ostreatus* on MANAE-agarose (Brugnari et al.2018).

Effect of storage and reuse on Immobilized laccase activity

Immobilized laccase retained 100 and 78.29% of its initial activity after storage for 24 and 30 day at 4°C, respectively (Figure 5), and its retained 100 and 80.47% of its activity for 21 and 30 continue usage, respectively (Figure 6).

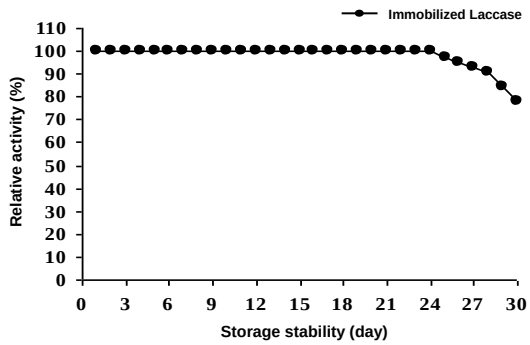

Figure (5): Effect of storage stability on immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads through 30 day at 4°C.

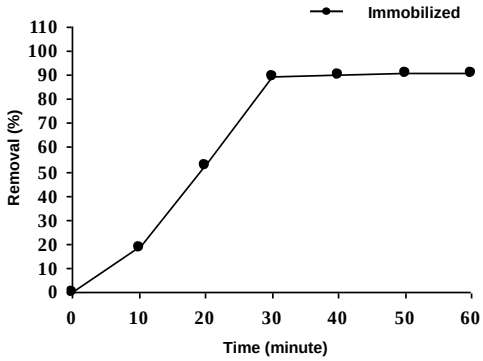

Figure 6: Effects of reuse (cycle) on immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads.

The stability of storage and number of reuse of immobilized enzyme are considered one of the important economic parameter for immobilization process and use it in specified application is successfully and effectively (Alsoufi and Aziz, 2020; Alsoufi, 2019).

In this regard, laccase from *T. versicolor* on magnetite nanoparticles of Fe_3O_4 retained 95.1% of its orginal activity after 10 weeks of storage at 4°C (Wang et al.2020), while, the enzyme from *B. atrophaeus* on the same matrix retained about 73, 60 and 26% of its initial activity after 5, 10 and 20 cycle, respectively (Narnoliya et al.2019), and the immobilized laccase from *M. thermophila* on epoxy-functionalized silica dropped to 61% of its orginal activity after 5 cycle of use (Mohammadi et al.2018). Whilst, Brugnari et al. (2018) reported that the enzyme from *P. ostreatus* on MANAE-agarose kept 80 and 70% of its initial activity after 40 and 170 day of storage at 4°C, respectively, also, Ilk et al. (2016)

observed that enzyme on nano composites lost 35% of its original activity after stored at 4°C for a 30 day, and retained 77% of its activity at the end of 10 cycle of use, while, Aydemir and Güler (2015) found that the storage stability of enzyme from *T. versicolor* on magnetic chitosan clay retained about 55% after 6 week at 4°C, and after the 10th use, the residual activity was found to be 76%.

Bioremediation of phenolic compounds from orange juice

The treatment of orange juice by immobilized laccase lead to increase in transmittance to be 124.6% and color was reduce to be 33.7% (Figure 7), therefore, the transmittance and color was improved after this treated that beneficial for use in removal of phenolic compounds from juice and other applications (Ilame and Singh, 2015).

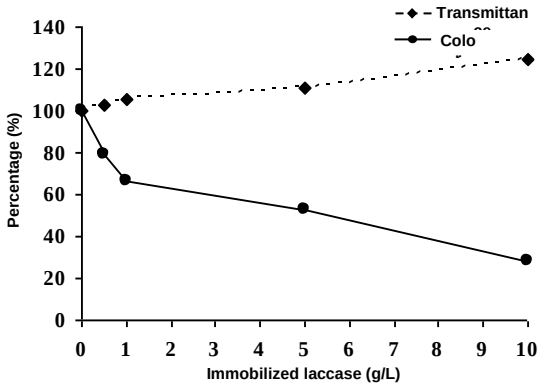

Figure 7: The change in transmittance and color for treated of orange juice by immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads.

The estimation of the require time for removal of phenolic compounds from juice by enzyme showed that the treated for 10, 20, 30, 40, 50 and 60 min lead to removed 18.37, 52.79, 89.16, 90.08, 91.02 and 91.06% of phenol compounds respectively (Figure 8).

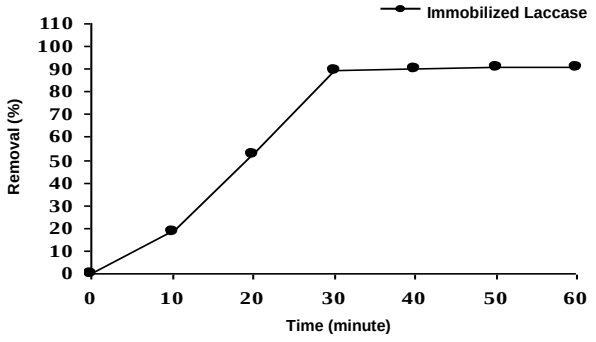

Figure 8: The require time for bioremediation of phenolic compounds from orange juice by immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads.

The of the results refer that the light transmittance of orange juice after heating at 70°C for 30 min, freeze at -18°C and thawing was higher than that before treated with immobilized laccase, in addition to the presence of sediment in non-treated juice. The stability of orange juice treated with immobilized laccase after heating, freezing and thawing was more than non-treated juice (Figure 9).

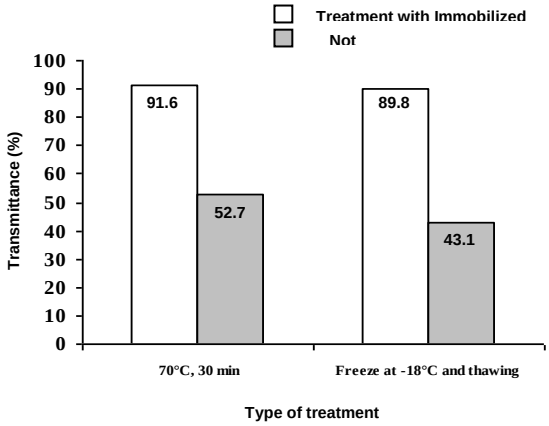

Figure 9: The light transmittance of treated orange juice by immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads after heating at 70°C for 30 min, freeze at -18°C and thawing.

Immobilized laccase can be used to clarify fruit juices due to its ability for removal of phenolic compounds by oxinidation to *o*-quinones, which lead to removed this compounds that cause haze and sediment in fruit juices (Yin et al.2017; Alsoufi, 2018). In this regard, a good deal of research referred to that. The clarification of apple juice by Immobilized laccase from *T. versicolor* on magnetite nanoparticles of Fe_3O_4 lead to enhanced of the light transmittance by 20.2%, decreased of color by 33.7%, reduced of phenolic compounds by 16.3%, and the treatment produced an apple juice have a good freeze-thaw and thermal stability (Wang et al.2020). the immobilized laccase from *B. atrophaeus* on magnetite nanoparticles of Fe_3O_4 was tested on juice of banana pseudo-stem, sorghum stem and apple fruit juice to reduction about 41-58% of phenol, decolorization 41-58% and reduction about 50-59% of turbidity (Narnoliya et al.2019). Brugnari et al. (2018) use immobilized laccase from *P. ostreatus* on MANAE-agarose maintained for degradation of bisphenol A (BPA). Lettera et al. (2016) refer that the clarification of orange, pomegranate, apricot, peach, cherry and apple juice by immobilized laccase from *P. ostreatus* on epoxy activated poly (methacrylate) beads lead to reduction up to 45% of phenolic compounds, and improved sensory profile of juice. The laccase-glutaraldehyde-coconut fiber reduced 61, 29 and 40% of color, turbidity and phenolic compounds of apple juice, respectively (Bezerra et al.2015).

CONCLUSION

This study showed the ability of immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads in removal of phenolic compounds from orange juice with high efficiency and improvement of the characteristics of juice.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEDGEMENT

This study showed the ability of immobilized laccase from *Agaricus bisporus* by calcium alginate-bentonite beads in removal of phenolic compounds from orange juice with high efficiency and improvement of the characteristics of juice.

AUTHOR CONTRIBUTIONS

Mohammed A Alsoufi: designed the study, collection of data, analysis, lab experimental, interpreted the data and drafted the article.

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