

Production of phenolic antioxidants from apple residue using *Rhizopus oligosporus*

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Abstract

In this research the 2 sub varieties of *Rhizopus oligosporus* was used for production of antioxidative bioactive compounds from whey protein concentrate (WPC) and apple pomace. Total phenolic compounds, free radical scavenging ability by DPPH and antioxidant protection factor were evaluated in 48 determined treatments. Effect of Incubation temperature in three levels 23,27,31°C, incubation time at 3,8,13 days, and Apple pomace/WPC ratio at three levels of 50:50, 70:30, 90:10 were studied. The polyphenol content, percent of DPPH inhibition and APF of the extracts was found to be in the range of 6.01-11.12 mg GAE/g DW, 65.8%-95.2% and 1-1.55 of samples respectively, depending on the ratio of Apple/WPC, time of incubation, type of fungi and temperature. The highest obtained efficiency was related to *Rhizopus oligosporus* PTCC NO.5287 and the results showed that the time of incubation had the most effect on measured parameters.

Keywords Antioxidant, Apple pomace, *Rhizopus oligosporus*, Whey Powder

Introduction

Apple residue, as well as many other agricultural wastes, can cause serious environmental problems, since it accumulates in agro-industrial yards without having any significant industrial and commercial value. Solid-state bioprocessing consists of the utilization of water-insoluble substrates for microbial growth and it is usually carried out in solid or semi-solid systems in the near absence of water [2]. Phenolic compounds widely exist in fruits and vegetables. There are more than 8000 phenolic structures that have been identified and this group of substances is considered to be one of the most numerous and widely distributed in the plant

kingdom [9]. As carcinogenic properties have recently been reported for some synthetic antioxidants [10]. *Rhizopus oligosporus*, also known as food-grade fungus that has been successfully grown in solid substrate systems using fruit residues as substrates [6]. Previous studies have shown that *R. oligosporus* is able to produce high amounts of β -glucosidase when grown in solid-state systems and is capable of hydrolyzing phenolic glycosides. This glycosidase-linked release is suggested to play an important role in the development of antioxidant functionality of these compounds [6].

The most abundant polyphenols present in apples are chlorogenic acid, phloretin glucosides and quercetin glucosides. Other polyphenolic compounds, such as catechins and procyanidins, have also been identified, but are present in relatively small amounts. While the potential of apple pomace as a source of polyphenols seems clear, there is less information on potential strategies for the recovery of these compounds. Most of the polyphenolic compounds are present in a bound form with carbohydrates, such as glycosides in nature. This bound nature of polyphenolics as glycosides reduces their ability to function as good antioxidants [5].

Material and methods

The material was autoclaved at 121 °C for 10 min and each flask was inoculated with ten 0.5 in. × 0.5 in. of activated *R. oligosporus* mycelium from a PDA plate cultivated for 7–10 days. Ten milliliter autoclaved distilled water was added to each flask, which were then covered with gauze layers and incubated. After growth, 100 ml of distilled water was added into of each fungus-pomace flask and the culture was homogenized in a Waring blender for 1 min followed by centrifugation at 11,000 × g at 4 °C for 30 min.

The supernatant was filtered through Whatman No. 1 filter paper.

A. *Total phenolics*

The concentration of total phenolics compounds in water extracts was measured using an assay modified by Shetty et al. One milliliter of the supernatant was mixed with 1ml of ethanol 95%, 5ml of distilled water and 0.5 ml of 50% (v/v) Folin–Ciocalteu reagent and immediately vortexed. After 5 min, 1ml of sodium carbonate was added and allowed to stand for 60 min in the dark. Samples were mixed again and their absorbencies measured at 725 nm against an ethanol 95% blank. The absorbencies were converted to micrograms of gallic acid per milligram of the sample. A calibration curve was built using (±)catechin as standard.

B. *Free radical scavenging activity of polyphenolic compounds*

The effect of polyphenol extracts on DPPH radical was determined according to the method by Brand-Williams, Cuvelier, and Berset (1995). A 100 μ M solution of DPPH in methanol was prepared and polyphenol extract (200 μ l) was mixed with 1 ml of DPPH solution. The mixture was shaken vigorously and left in the dark at room temperature for 20 min. The absorbance of the resulting solution was measured at 517 nm. The control contained all the reagents except the polyphenol extract. The capacity to scavenge DPPH radical was calculated by following Equation:

$$\text{Scavenging activity} = [1 - (A_s/A_0)] * 100$$

where A_0 is the absorbance at 517 nm of the control and A_s is the absorbance in the presence of polyphenol extract. The results were plotted as the percentage of scavenging activity against concentration of the sample. The half-inhibition concentration (IC_{50}) was defined as the amount of sample in dry weight required for 50% of free radical scavenging activity. The IC_{50} value was calculated from the plots as the antioxidant concentration required for providing 50% free radical scavenging activity.

C. *β -Carotene oxidation assay*

One milliliter of 0.2 mg/ml of β -carotene in chloroform was pipetted in to a round-bottomed flask covered with aluminum foil. Chloroform was evaporated at 40 °C for 10 min. The β -carotene adhered to the sides of

the flask was scraped and dissolved in 20 μ l of linoleic acid (Sigma Chemical Co., St. Louis, MO) and 184 μ l of Tween 40TM. To this mixture, 50 ml of 50 mM H_2O_2 was added and vigorously mixed until completely homogenized. Each test tube containing 100 μ l of sample extract received 5ml of the emulsified mixture and vortexed. The tubes were incubated at 50 °C (water bath) for 30 min. Once removed, the samples were vortexed once again and their absorbencies measured at 470 nm. Control samples were analyzed having 100 μ l of water in place of the extract. The antioxidant protection factor (APF) was expressed as (sample absorbance at 470 nm)/(control absorbance at 470 nm).

D. *Statistical analyses*

In this research, statistical plan was split design with 8 whole plots and for data analysis was used of REML method at $\alpha=0.05$.

E. *Results and discussion*

The polyphenol content in apple pomace was determined. It was found that the polyphenol content increased from 5.53 to 11.6 mg GAE/g dry weight (DW) of samples. Antioxidant activities of polyphenol extracts were tested using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical methods and the IC_{50} ranged from 23.24 to 48.42 μ g DW sample. APF of polyphenol extracts increased from 1 to 1.55 depending on the culture condition.

1. *Total phenolics*

During first 4 days of growth, treatments showed a similar trend. This low phenolic content observed during the early stages of growth suggests that most natural phenolics were in bound form and only a relatively small part was in free phenolics form (soluble). The phenolic compounds in the extracts are more often associated with other biomolecules, such as protein, carbohydrates, lipids, terpenes, chlorophyll and other organic compounds. After 6 days of growth phenolic content increase with increasing in apple/whey until 50/50 in substrate. Results of total phenolics with desirability curve showed in fig.1.

2. *Free radical scavenging activity*

The free radical scavenging activity of polyphenol extracts from apple pomace with different condition was measured as IC_{50} value and the results are shown in fig.2. DPPH method is a widely used method for antioxidant activity studies. The method is based on

the reduction of alcoholic DPPH solutions at 517 nm, in the presence of a hydrogen donating antioxidant and polyphenols have been reported to be potent

hydrogen donors to the DPPH radical because of their ideal structural chemistry.

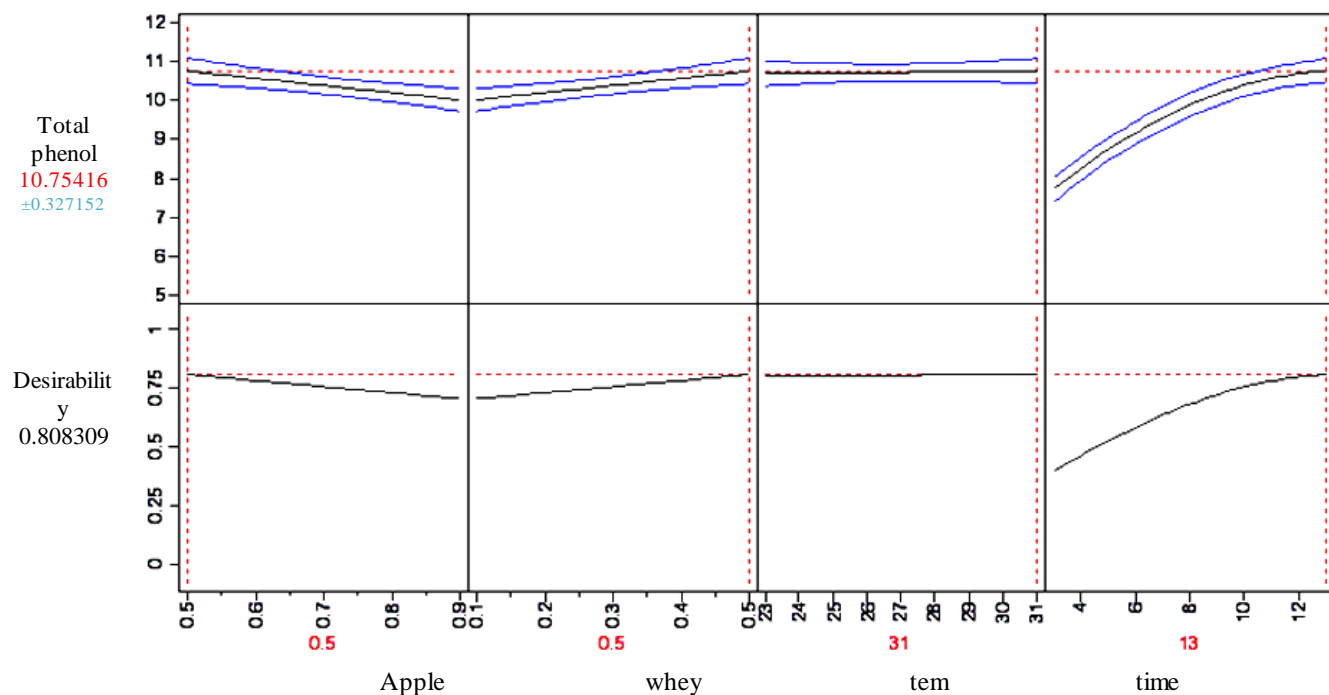


Fig. 1. Total phenolics content of treatments

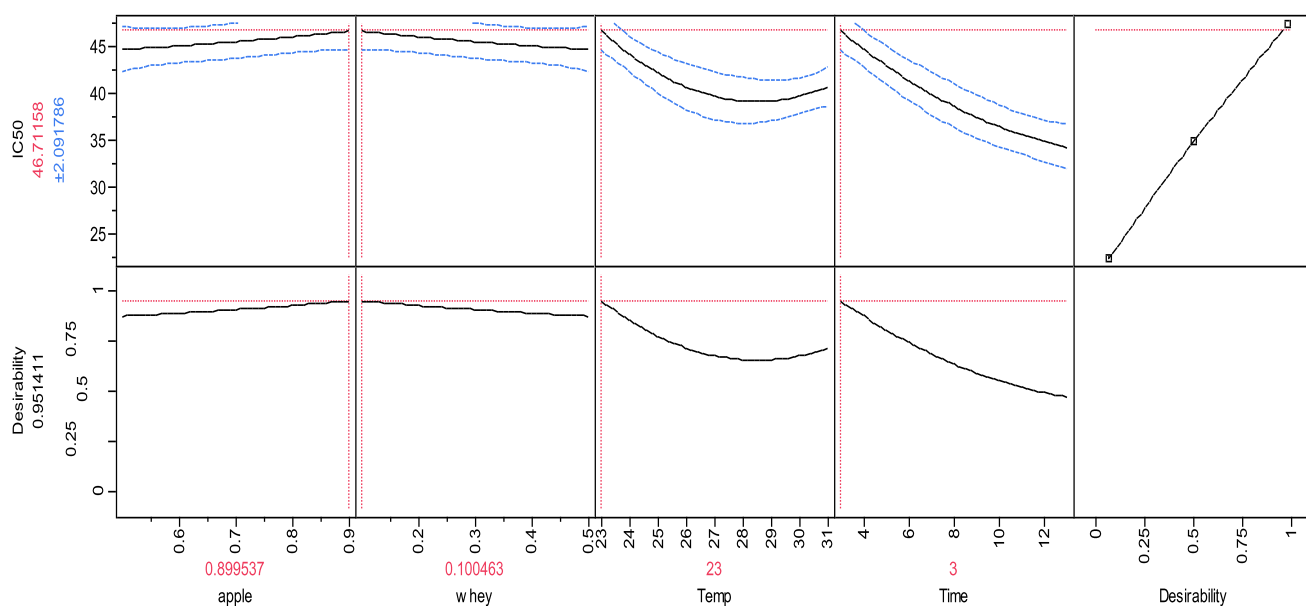


Fig.2. Radical scavenging activity of polyphenol extract

3. β Caroten assay

The result of APF was showed in fig.3. the time of incubation was highest effective on this factor and enhanced APF with increasing apple/whey until 50/50 in substrate during fermentation could be explained by a higher lipid solubility of the compounds released at this stage of the growth that could result in a higher activity at the lipid–water interface. This high antioxidant levels coincide with high total phenolics content. These results indicate that the antioxidant activity of the samples during later days of growth is higher than in the early growth (3 days). This tendency

is observed for treatments and indicates that in later stages the antioxidant phenolics mobilized are more hydrophobic in nature. A positive correlation was found between DPPH and β -carotene assays, which was statistically significant between treatments ($P < 0.05$) (Figs. 2 and 3). During the later stages of growth (13 days) the extracts have increased antioxidant activity in both assays, which could suggest both assays effective free radical quenching capacity and antioxidant protection.

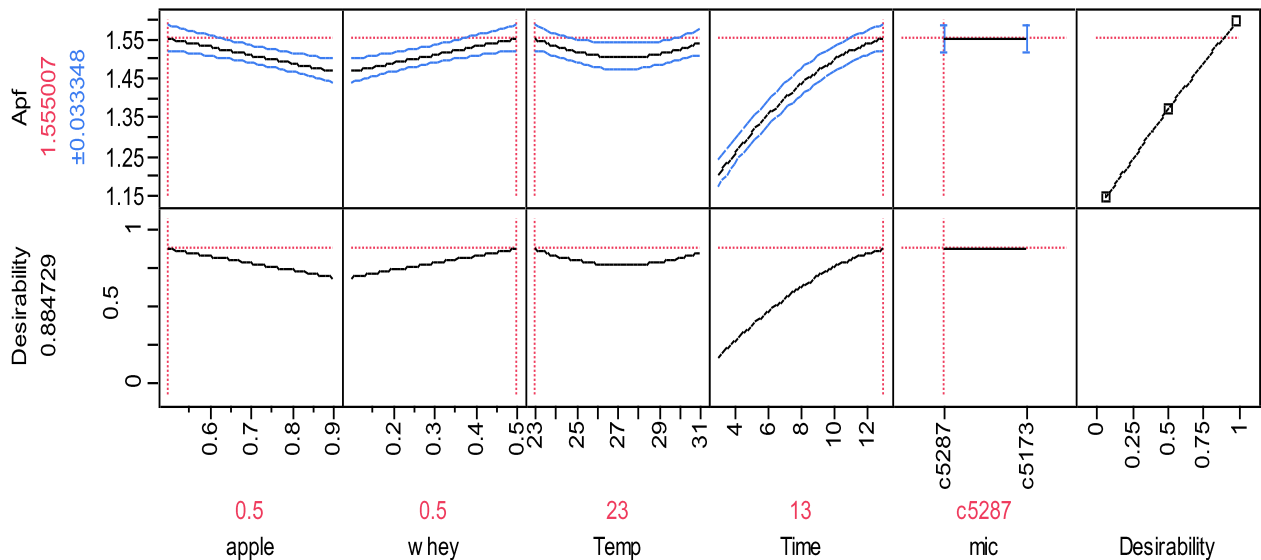


Fig.3. Antioxidant protection factor (APF) for treatments

F. Conclusions

A significant positive correlation ($P < 0.05$) between total phenolic content and DPPH inhibition and APF with growth of *Rhizopus oligosporus* in time of incubation. The bioconversion of apple residue mixed with whey powder lead to enhanced total phenolic content, mainly for the apple/whey (50/50) treatment. The moderate final total phenolic content of (90/10) treatment can be caused by nutritional limitations, since apple does not have high nitrogen

content. This approach helps to enhance the value of apple residue, not only its commercial value, but can also provide a source of health-relevant compounds. However The present study showed that the enrichment of apple pomace with phenolic antioxidants can be carried out by solid-state fermentation using white rot fungus, *Rhizopus oligosporus*. Solid-state fermentation thus, not only resulted in value addition to the apple pomace, but also improved the antioxidant activity.

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