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Impact of Bioprocessing on phenolic content & antioxidant activity of mung seeds to improve hypoglycemic functionality

Debasmita Maiti, Mala Majumdar*

Department of Biotechnology, Center for Postgraduate Studies, Jain University, Bangalore, India.

Corres.author: malamajumdar51@gmail.com

Abstract: Long-term type 2 diabetes can lead to numerous biological complications, such as hypertension and cardiovascular disease. Key enzymes involved in the enzymatic breakdown of complex carbohydrates, pancreatic α-amylase and intestinal α-glucosidase have been targeted as potential avenues for modulation of type 2 diabetes-associated post-prandial hyperglycemia. Mung (*Vigna radiata*) bean substrates optimized for phenolic content through solid-state bioconversion (SSB) by dietary fungus (*Rhizopus oligosporus)* were investigated for inhibitory activity against porcine pancreatic α-amylase and yeast α-glucosidase. SSB is a microbial bioprocessing method, characteristics of cereals and legumes. *R. oligosporus*-bioprocessed mungbean extracts possessed marked anti-amylase activity, but only slight anti-glucosidase activity. The anti-amylase activity of mung bean exhibits positive correlation with antioxidant activity. The results suggest that the dietary fungal bioprocessing improves the anti-diabetic potential of mungbean extracts, potentially through modulation of the phenolic profile of the extract, and further suggest that enzyme inhibitory activity may be linked to phenolic antioxidant mobilization during bioprocessing. The significance of food-grade, plant-based enzyme inhibitors for modulation of carbohydrate breakdown and control of glycemic index of foods in the context of preventing hyperglycemia and diabetes mellitus complications in the long term is discussed.
Key words: Solid state bioconversion (SSB), *Rhizopus oligosporus, In vitro*, Antioxidant activity, Hypoglycemic potential.

Introduction

Prevalence of non-insulin dependent diabetes mellitus (NIDDM), a chronic disease characterized by hyperglycemia, is rapidly rising worldwide at an alarming rate and is emerging as a major health problem in India (1). It is projected that NIDDM may affect 366 million people globally by 2030 (2). It is now well recognized that the body's natural antioxidant defenses or by dietary antioxidants supplements reduce the risks and prevent progression of chronic diseases (3). Synthetic antioxidants have side effects and they are carcinogenic (4). Hence, medicinal plants, which are rich in antioxidants, continue to play a major role in the treatment of NIDDM particularly in developing countries (5). *In vitro* studies suggest that vegetables, grains and seeds, which are rich in antioxidants, have protective effects against many diseases such as cancer, diabetes and cardiovascular diseases (6).

α-Amylase and α-glucosidase are key enzymes involved in starch breakdown to glucose in intestine. Inhibition of these enzymes can significantly decrease the postprandial hyperglycemia after a

mixed carbohydrate rich diet and can be an important strategy in the management of hyperglycemia linked to NIDDM (7). Main drawbacks of currently used inhibitors, for example acarbose, are the side effects such as abdominal distention, flatulence and possibly diarrhea (8). Presently, attention has been focused on natural enzyme inhibitors from plant sources, which can be used as an effective therapy for postprandial hyperglycemia with minimal side effects (9). They are rich in phenolic substances and proteins, which interact with digestive enzymes thereby modulating their activity (10).

Solid State Bioconversion (SSB) is a simple microbial bioprocessing of a solid food substrate that acts as a physical support and source of nutrients in the presence of low free liquid (11). *Rhizopus oligosporus* is a food-grade fungus that has been widely used in solid-substrate bioconversion systems to produce value-added food products (12) and is effective for substrates such as soya, fava bean, cranberry pomace, and pineapple (13**)**. This approach would increase the phenolic content and antioxidant activity which will enhance the potential health-relevant functionality of fungal processed seed and legumes.

Mung bean (*Vigna radiata*) is an excellent source of vitamins, minerals and protein and essential amino acid (14). Recent research indicates that mung bean consumption produces small increase in blood glycemic index in humans, making it an attractive option for diabetic patients (15). It is reported to modify glucose and lipid metabolism favorably in rats (16).

Patients with diabetes mellitus are likely to develop certain complication such as retinopathy, nephropathy and neuropathy as a result of oxidative stress and overwhelming free radicals (17**)**. Recent interests in the use of non-vitamin antioxidants such as flavonoids and polyphenols in reducing the negative effect of oxidative stress and free radicals in diabetic patients (18**)** are developed. Hence, food sources with hypoglycemic effect and high antioxidant activity such as mung bean are beneficial for diabetics (19).

The aim of the present study is to evaluate the potential of bioprocessing by food-grade fungus, *R. oligosporus*, to improve the phenolic content, antioxidant activity as well as associated α-amylase and α-glucosidase inhibition.

Materials and methods

Seeds and microorganism*:*

Plant seeds (Mung- *Vigna radiata)* were procured from Namdhari seeds, Bangalore, Karnataka, India. Specimen samples have been deposited at Jain University herbarium (voucher no. 3412). These seeds were shade dried at room temperature (30℃) to constant weight for 5 days and powdered. *Rhizopus oligosporus* (stain no-MTCC 556) was procured from IMTECH, Chandigarh, India and was maintained on potato dextrose agar plates and sub-cultured monthly. The fungal culture at active sporulating stage (which is approximately three weeks of culture at room temperature) was used in this study (20).

Solid state bioconversion (SSB)

10 g of dry seed powder along with 25 ml distilled water were taken in 250 ml Erlenmeyer flasks and autoclaved. Flasks were inoculated with fungal spores and incubated at room temperature (30℃) for 10 days under static condition. Sample (2ml), withdrawn at two-day intervals, was homogenized and centrifuged at 15,000 g at 4 °C for 20 min. The supernatant was then filtered through a Whatman No. 1 filter paper (20). Filtrate was used for further analysis.

Determination of total protein content

The total protein content of the bioprocessed seed extracts were determined by Lowry's method using BSA as standard and absorbance was measured at 660 nm All determinations were carried out in triplicates (21).

Determination of total phenol content

Total phenol content of seed extracts was determined using the method of Mcdonald et al. (22), with slight modifications. Gallic acid was used as the standard for the preparation of calibration graph (0.025-0.4 mg/ml) and absorbance was measured at 765 nm. Phenol concentration in the extracts was calculated in terms of gallic acid equivalent and was expressed as mg TPC/g DW of seed powder.

Measurement of DPPH Radical-scavenging activity

Antioxidant activity of seed extracts was determined on the basis of the scavenging effect on the stable DPPH (1, 1-diphenyl-2-picrylhydrazyl) free radical activity (23). DPPH solution was freshly prepared and kept in the dark at 4°C. To 3 ml of 60 µM DPPH, 100 µl of seed extracts were added and the mixture was shaken vigorously and left in the dark at room temperature for 30 min. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The radical scavenging activities, of tested samples, were expressed as percentage of inhibition (24).

α-Amylase inhibition

Amylase activity was measured as described by Bernfeld (25) with the following modifications. The assay mixture contained the enzyme, 30 μmol of sodium phosphate buffer pH 6·9 and 10·5 μmol of NaCl in volume of 1·5 ml. The reaction was initiated by the addition of 0·5 ml of 1% starch solution. After 5 min incubation at 37°C, the enzyme action was arrested by 1·0 ml of dinitrosalicylate reagent. The solution was kept in a boiling water bath for 10 min, cooled and diluted to 11·0 ml with water. The colour was measured at 540 nm. A standard curve was generated using D-(+)-maltose monohydrate. Inhibition of α-amylase activity was expressed as amylase inhibition (AI) index values, which was defined as the ratio of the amylase activity of the control (enzyme alone) to that of the enzyme (26).

α- Glucosidase inhibition

The inhibitory activity of bioprocessed seed extracts against yeast α-glucosidase was determined by measuring the liberation of *p*-nitrophenol by α-glucosidase, with *p*-nitrophenyl-α-D-glucopyranoside (PNP) as the substrate. To assess the inhibition, experiments were carried both in the presence and absence of seed extracts. 0.2mL of 0.2 mM PNP substrate (distilled water for control) and 0.2mL of the enzyme was added to seed extract mixture. This reaction mixture was incubated for 30min in at 30°C. The reaction was stopped by addition of 0.6mL of 1M sodium carbonate and absorbance was determined in a spectrophotometer at 405 nm (26). Data was reported as α- glucosidase inhibition (aGI) index values, defined as the ratio of the α-glucosidase activity of the control (enzyme alone) to that of the enzyme/ seed extract mixture.

Statistical analysis

Experiments were performed in triplicates. The average values of the experiments for each day with standard deviations are reported. Statistical analysis was performed using MS- Excel software.

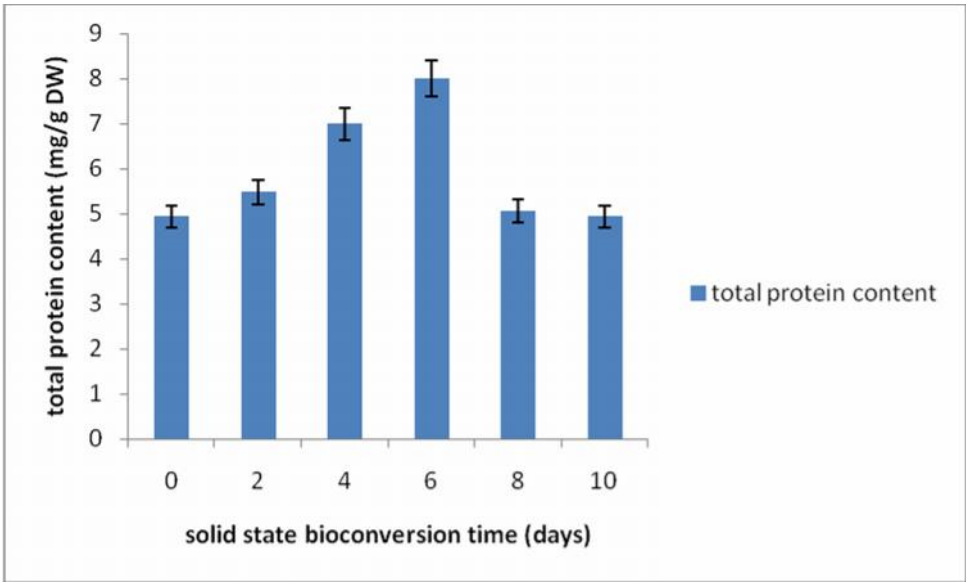

Fig: 1 Effect of SSB on total protein content in different seed extracts

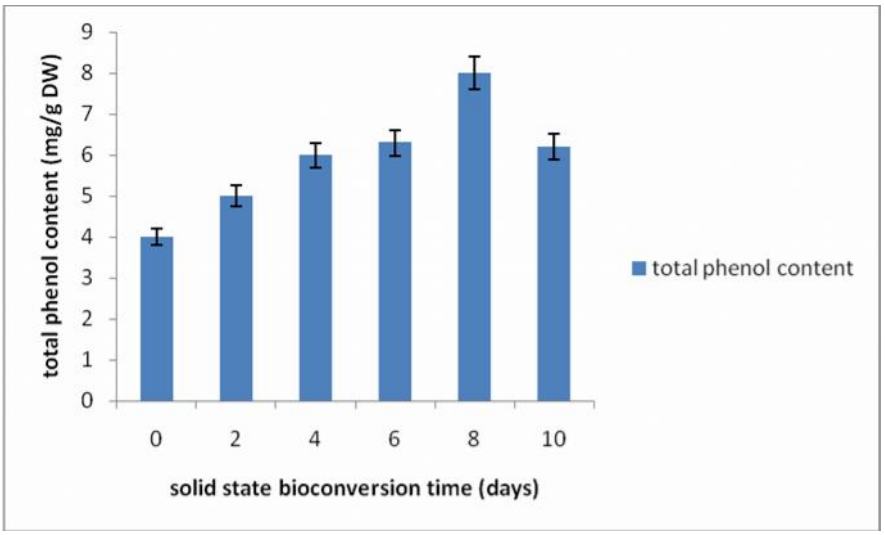

Fig: 2 Effect of SSB on total phenolic content of seed substrates

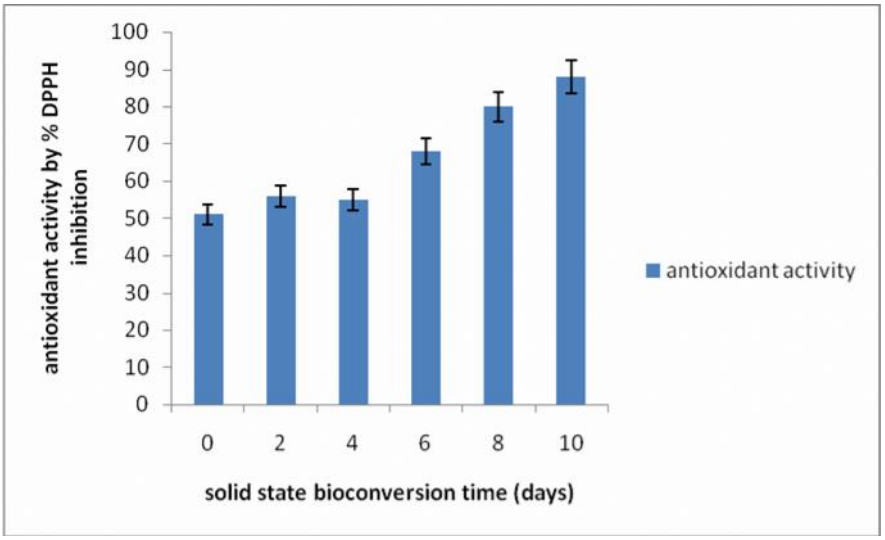

Fig: 3 Antioxidant activity of seed substrates during SSB by *R. oligosporus* by DPPH radical inhibition method.

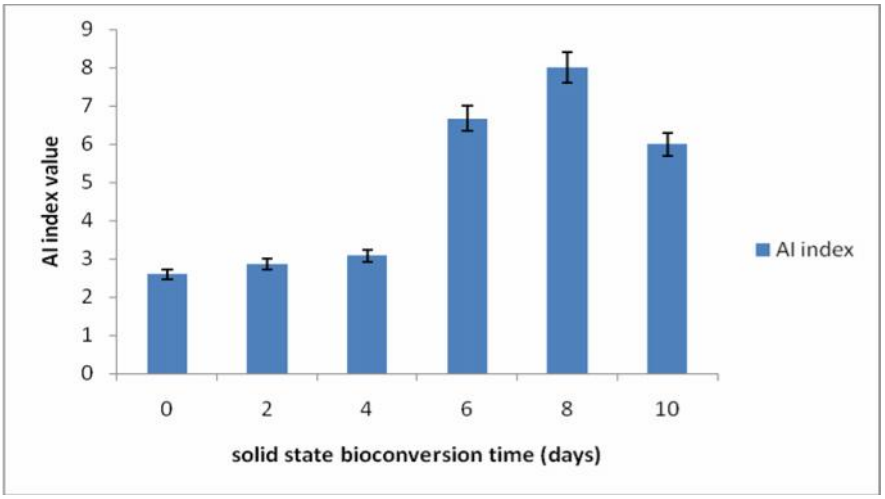

Fig: 4 AI index in bioprocessed seeds for alpha amylase inhibition

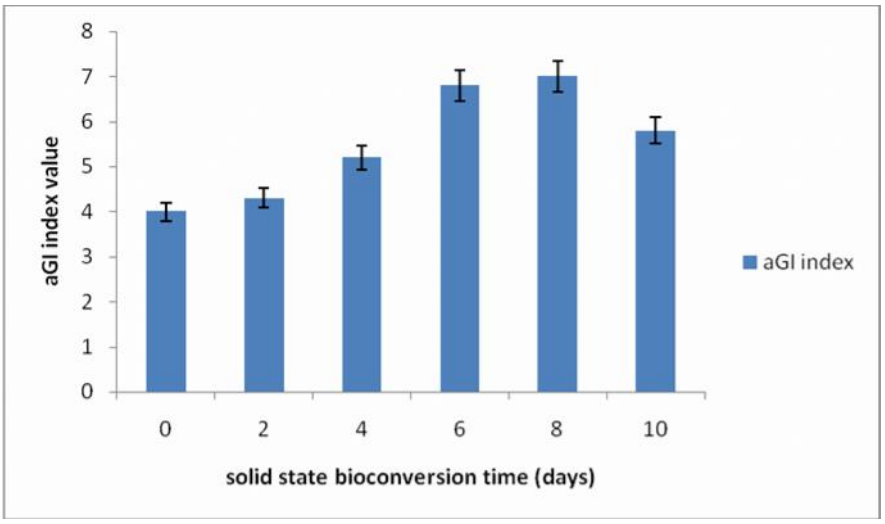

Fig: 5 α-Glucosidase inhibition (aGI) by extracts of bioprocessed seeds by *R. oligosporus*

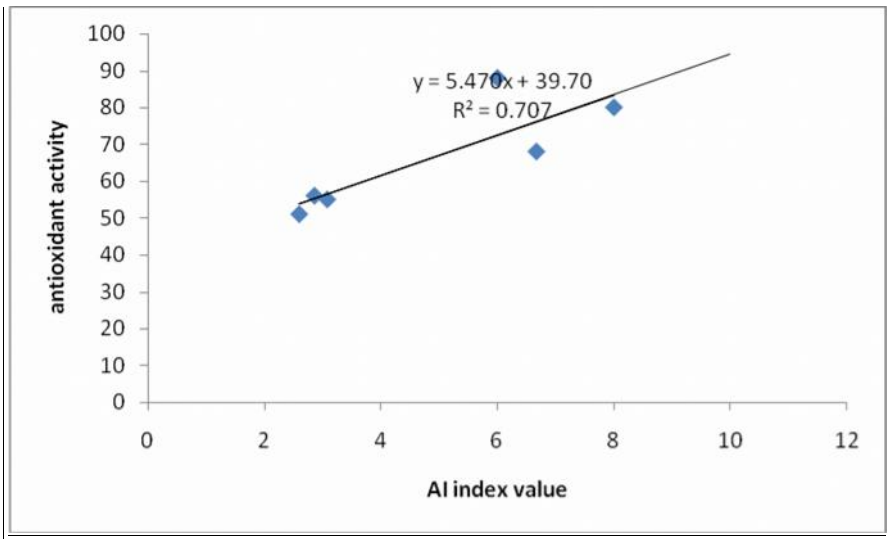

Fig: 6a Correlation between AI index and antioxidant activity

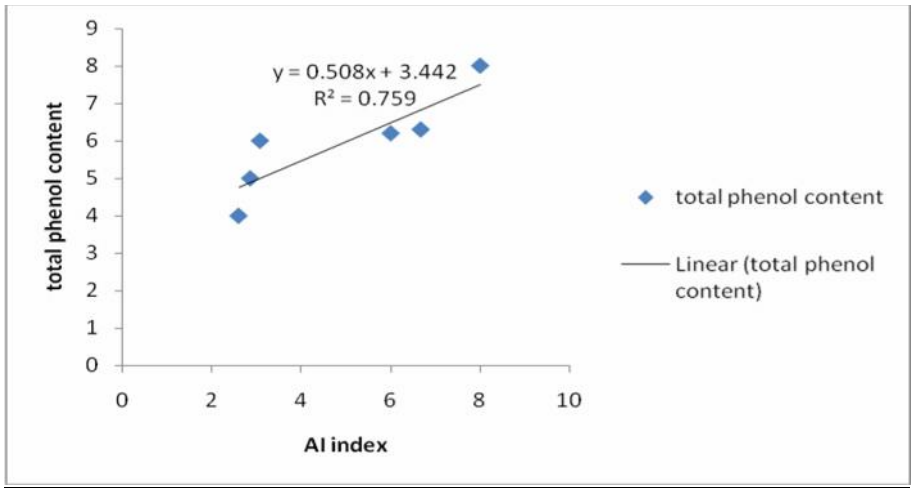

Fig:6b Correlation between TPC and AI index

Results and Discussion

During SSB of mung bean substrates by *R. oligosporus* the growth of the fungus was rapid from day 6 of inoculation and efficiently colonized the entire substrate by day 10. The protein content of the inoculated seed substrates, which is an indicator of the fungal growth, increased with incubation time (Fig.1). Protein content has increased in bioprocessed seeds: On 6^{th} day, the protein content has increased by 62.27% in mung (Fig.1), which indicated an efficient colonization of the fungus on the seed substrates, hence phenolic mobilization by the fungus was investigated. The changes in the total phenolic content due to mobilization by *R. oligosporus* on the seed substrates are shown in fig 2. The phenolic content doubled by day 6 in case of mung (4 mg/g DW – 8 mg/g DW), when an increase in protein content was detected, indicating an active phenolic mobilization by the fungal enzymes (20) (fig.2). This enhanced phenolic content can improve the health-relevant functional value of the seed extracts.

Phenolic compounds are known to exhibit free-radical scavenging (antioxidant) activity, which is determined by their reactivity as hydrogen or electron donors, the stability of the resulting antioxidant-derived radical, their reactivity with other antioxidants and their metal chelation properties (27). The antioxidant activity of mung bean as measured by DPPH inhibition method were moderately high (51%) and gradually increased to 88% on day 10, when the total phenolics content were only moderate (fig: 3).

α-Amylase is responsible for cleaving starch during digestive process, which is important to managing postprandial blood glucose levels. Mild α-amylase inhibitors have the potential to be therapeutic agents in the treatment of diabetes and obesity, since they are potentially positive modulators of these postprandial blood glucose levels (20). Amylase inhibitors are also known as starch blockers because they contain substances that prevent dietary starches from being rapidly absorbed by the body. A bi-functional inhibitor from seed extracts which exhibit inhibitory activity towards trypsin-like and α-chymotrypsinlike serine proteinases as well as against α-amylases has been reported (28). The α-amylase inhibitory activity of the bioprocessed mungbean extracts was moderately high during early incubation (days 0–2) (Fig.4). Higher inhibition was observed during days 4–10 which correlate with higher phenolic levels (29). It is suggested that the mechanism of inhibition of the glycolytic activity of α-amylase may occur through the direct blockage of the active center or at several sub sites of the enzyme as also suggested for other plant-based inhibitors (29, 30**)**. Extracts of *R. oligosporus*-bioprocessed mungbean had the strongest anti-amylase activity, specifically after 6-8 days of culture time(AI index value = 8 ± 0.02) (Fig.4). Whereas the anti-glucosidase activity is seen comparatively less in mung bean seed extracts (aGI index value = 7 ± 0.006) (Fig.5).

Several studies have reported an association between TPC, antioxidant activity and starch hydrolyzing enzymes such as α amylase and α glucosidase (31,32,33). Relationship of TPC/antioxidant and α amylase enzyme inhibition was assessed for mung. There was marginal correlation between TPC and antioxidant activity (measured by DPPH method). This is also reflected that amylase inhibition was better correlated with both antioxidant activity and TPC for mung bean as compared to TPC/antioxidant activity (Fig. 6). These results show the presence of non phenolic antioxidants in bioprocessed seeds, which may inhibit these enzymes. Surprisingly, there was no significant correlation of glucosidase inhibition either with TPC or antioxidant activity.

Conclusion

The major implication of this research is that SSB can be an efficient strategy to improve the phenolic content of mung bean extracts with associated enhancement of health-linked functionality. Results indicate that during the early stages of bioprocessing there is enhancement of total phenolics and antioxidant activity in these seed extracts. This increase also correlates to higher α-amylase inhibition and α-glucosidase inhibition. It is proposed that the improvement in functionality is due to the fungal enzymatic hydrolysis of the seed substrates.

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