Original Article

Improved α-amylase and *Helicobacter pylori* inhibition by fenugreek extracts derived via solid-state bioconversion using *Rhizopus oligosporus*

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The present research investigated the enrichment of fenugreek (*Trigonella foenum graceum*) seed substrate with phenolic antioxidants and L-DOPA via fungal-based solid-state bioconversion (SSB) system. This approach using food grade fungus *Rhizopus oligosporus*, was chosen because it has been demonstrated to be effective in other seed and food substrates for improving health-relevant functionality and has long history of use for food processing in Asia. The protein content and β-glucosidase activity of the substrate which reflects fungal growth, increased with incubation time in conjunction with enhanced phenolic content and also suggested its possible involvement in phenolic mobilization. The antioxidant activity assayed by β-carotene bleaching and DPPH free radical scavenging methods both indicated high activity during early growth stage (days 4-6) followed by reduced activity during later growth stage (days 8-20). A direct association between higher phenolic contents during early growth stage (days 4-6) and antioxidant activity suggested a link to mobilization of polymeric and hydrophobic phenolic forms. The L-DOPA content of the fenugreek extract fluctuated during the course of bioconversion with higher levels during days 6-10 (1.5-1.7 mg/g DW). The SSB process substantially improved the *in vitro* porcine α-amylase inhibition activity by 75 % on day 4 which correlated to higher levels of total phenolics and related antioxidant activity of the extracts. The high α-amylase inhibitory activity also coincided with high L-DOPA content on day 6. These results have implications for diet-based diabetes management. The same bioconversion stage had *Helicobacter pylori* inhibitory activity, which has implications for ulcer management.

Key Words: fenugreek, α-amylase inhibition, type 2 diabetes, solid-state bioconversion (SSB), *Rhizopus oligosporus*, β-glucosidase, phenolics, antioxidant activity, L-DOPA (levo-dihydroxy phenylalanine), superoxide dismutase, antioxidant protection factor, *Helicobacter pylori* inhibition

INTRODUCTION

Fenugreek (*Trigonella foenum graceum*) is noted for its several pharmacological properties especially its hypoglycemic effects.[1, 2] The seeds are rich in proteins and contain the unique amino acid 4-hydroxyisoleucine, which is one of the active ingredients for blood glucose control by inducing insulin release in both rats and humans.[3, 4] Type 2 diabetes is increasing globally with ensuing concern about the cost of management and control. Many oral hypoglycemic agents, such as biguanides and sulfonylurea are available along with insulin for its treatment[5] but these synthetic agents can produce serious side effects including abnormal colon function. The use of synthetic α-glucosidases inhibitors such as acarbose, cause adverse side effects such as abdominal distention due to the excessive inhibition of pancreatic enzymes, resulting in the abnormal bacterial fermentation of undigested carbohydrates in the colon.[6] Hence, research on the development and utilization of antidiabetic plants with mild inhibition of pancreatic enzymes is beneficial.[7, 8] These plants contain phenolic substances and proteins, which interact with digestive enzymes thereby modulating their activity.[9]

Further, an interesting association between diabetes and dopaminergic functioning in rats suggest that hypoglycemic foods with increased levo-dihydroxyphenylalanine (L-DOPA) content are desirable.[10-12] It is the precursor of the neurotransmitter dopamine, used in the management of Parkinson disease.[13] Previous studies indicated that fenugreek sprouts are rich in phenolics and L-DOPA, which improved with elicited priming.[14]

Helicobacter pylori is *a* bacterial pathogen that persistently inhabit the human stomach. Colonization by the bacterium induces inflammation of the gastric mucosa that may progress into peptic ulcer diseases or adenocarcinoma.[15] Antibiotic treatments do not always inhibit the bacterium and it has side effects with potential for antibiotic resistance. Earlier research indicated that clonal oregano extracts rich in plant phenolics with related antioxidant activity have moderate inhibition.[16]

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Solid-state bioconversion (SSB) refers to the growth of microorganisms especially fungi on solid substrate with minimal presence of free liquid between substrate particles. Hence, fungal bioprocessed foods rich in phenolics such as fenugreek could be an effective additional strategy for diet-based management of *H. pylori* infections coupled to reducing other oxidation-linked diseases. The primary objective of such bioprocessing is for the modification of the organoleptic and nutritional properties of the substrate to produce value-added products. The objective of this research was to investigate the potential of SSB system that can mobilize the conjugate forms of phenolic precursors naturally found in fenugreek. To this end, we chose food- grade fungus *Rhizopus oligosporus*, demonstrated to be effective in other substrates such as fava bean, cranberry pomace, and pineapple.[17-19] The rationale was that this approach would increase the phenolic content, antioxidant activity and L-DOPA content, which will enhance the potential health-relevant functionality of fungal processed fenugreek. We believe that the spectrum of phenolic compounds with associated higher antioxidant function that are mobilized during solid-state bioconversion, would confer antimicrobial activity with reduced potential for development of antimicrobial resistance due to the different modes of action of various individual phenolics in the profile. Similarly, it may also confer improved amylase inhibition activity with enhanced host antioxidant function for potential Type 2 diabetes management.

MATERIALS AND METHODS

Microorganism and solid state bioconversion

The SSB system followed was similar to the regular solid substrate bioconversion followed in Tempeh production.[20] *Rhizopus oligosporus* was originally isolated from an un-pasteurized Tempeh product in our laboratory. The fungus was maintained on potato dextrose agar plates and sub-cultured monthly. A fungal culture at active sporulating stage (approximately 3 week old culture at room temperature) was used in this study.

Dry seeds of fenugreek (*Trigonella foenum-graceum*) were purchased from Asian-American Groceries, Hadley MA, USA. For the SSB studies, 10 g of finely ground fenugreek powder was taken in 250 mL Erlenmeyer flasks. Then 25 mL distilled water was added and the flasks were autoclaved at 121°C for 15 min. The fungal spores and mycelia from one petri plate were inoculated into 4 flasks, mixed well and incubated at room temperature for 20 days. The samples were extracted at two-day intervals for 20 days. One hundred milliliters of distilled water was added to the flasks and the culture was homogenized for 1 min using a Waring blender, and then centrifuged at 15,000 g at 4°C for 20 min. The supernatant was then filtered through a Whatman No. 1 filter paper.

Total phenolics assay

Total phenolics were measured as gallic acid equivalents.[21] One mL of the above fenugreek extract was transferred to a test tube and 1 mL of 95% ethanol; 5 mL of distilled water and 0.5 mL of Folin-Ciocalteu phenol reagent (Sigma Chemical Co, St. Louis, MO) were added. After an incubation period of 5 min 1 mL of 5% Na_2CO_3 was added, mixed well and kept in the dark for an hour. Then the samples were vortexed and the absorbance was measured at 725 nm using a UV spectrophotometer (Spectonic Genesys 5; Milton Roy Company, Rochester, NY). Phenolic content was estimated from a standard curve of gallic acid under similar assay conditions and reported as mg/ g DW (dry weight).

Antioxidant activity assay

The antioxidant activity of the fenugreek culture extracts was determined by two methods namely the β-carotene oxidation and DPPH (1,1-diphenyl-2-picrylhydrazyl) inhibition assays. The β-carotene oxidation model system as described by Hammerschmidt & Pratt, 1978 was followed.[22] The β-carotene solution was prepared by dissolving 10 mg of β-carotene in 50 mL of chloroform in amber colored flask to prevent light oxidation. One mL of this solution was pipetted to a flask covered with aluminum foil. Chloroform was then evaporated under vacuum at 40˚C for 5 min. Then the β-carotene was dissolved in 20 µL of linolenic acid and 184 µL of Tween 40 emulsifier. Then added 50 mL of H_2O_2 solution (176 µL H_2O_2 in 100 mL distilled water) and mixed thoroughly till the β-carotene was completely dissolved. To 100 µL of the phenolic extracts 5 mL of this prepared β-carotene solution was added. Control tubes had 100 µL of 95% ethanol. As soon as the emulsion was added the zero time absorbance was read at 470 nm. Subsequent absorbance readings were recorded after a 30 min incubation period in a 50˚C water bath. The Antioxidant Protection Factor (APF) was used to express antioxidant activity as a ratio of sample absorbance at 30 min to that of the control.

The antioxidant activity of the fenugreek culture extracts was also determined by the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay.[23] To 3 mL of 60 µM DPPH, 100 µl of fenugreek culture extract was added and the absorbance was monitored at 517 nm. The radical scavenging activity of the fenugreek extract was compared with the activity of an equivalent concentration of quercetin, a strong antioxidant standard and expressed as DPPH radical inhibition percent.

HPLC analysis of L-DOPA

Ten milliliters of the fenugreek culture extract was evaporated at room temperature and the residue was dissolved in 10 mL of buffer solution (32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM Na_2EDTA, 0.215 mM octyl sulphate pH 4). This solution was filtered through 0.45 µm disposable syringe filters (Schleicher & Schuell, Keene, NH). High Performance Liquid Chromatography (HPLC) was performed using an Agilent 1100 liquid chromatograph equipped with a variable wavelength detector. The analytical column was a reverse phase Supleco Discovery C18, 250 mm x 4.6 mm with a packing material of 5 µm particle size. The total composition of the mobile phase was 18% methanol and 82% buffer consisting of 0.01 M ammonium acetate at pH 5.4 at a flow rate of 1 mL/min. Tyrosine and catecholamine standards [L-DOPA, dopamine, norepinephrine and epinephrine (Sigma chemicals, St. Louis, MO)] were chromatographed separately and in a mixture. The sample was

chromatographed under the same conditions. Retention time and spectrum was compared with that of the standard L-DOPA. The amount of L-DOPA in the fenugreek culture extract was measured from the peak height obtained at 280 nm, computed automatically using Agilent Chemstation 4.0 and was expressed in terms of milligrams per gram dry weight.

Crude enzyme extraction and protein assay

A crude enzyme extraction was made by dialyzing 10 mL of the fungal bioprocessed fenugreek culture extract using Spectro/Pro membrane tubing (Spectral Medical Industries Inc., Houston, TX) against distilled water at 5°C for 24 h. The resultant clear liquid was used as crude enzyme solution after adjusting the same volume for each extract.

The protein content of the extracts was measured by the Bradford method.[24] This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue shifts from 465 nm to 595 nm, specific to when binding to protein occurs. The Bradford dye reagent (Bio-Rad protein assay kit II, Bio-Rad Laboratory, Hercules, CA) was prepared by diluting the commercial dye concentrate in a 1:4 ratio with distilled water. To 100 µL of the sample and blank (extraction buffer only) in test tubes 5 mL of the dye was added and incubated at room temperature for 15 min. The samples were mixed and the absorbance was read at 595 nm using a UV-VIS Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY).

β-glucosidase activity assay

The β-glucosidase enzyme activity was measured by the procedure described previously.[25] A standard reaction mixture contained 0.1 mL of 9 mM p-nitrophenol β-D glucopyranoside (pNPG), 0.8 mL of 200 mM sodium acetate buffer (pH 4.6) and 0.1 mL of enzyme solution. After 15 min incubation at 50°C, the reaction was stopped by addition of 1 mL of 0.1 M sodium carbonate and the released p-nitrophenol was measured at 400 nm. The standard curve was established using pure p-nitrophenol (Fisher Scientific Co., Fair Lawn, NJ). One unit of enzyme was defined as the amount of enzyme that releases 1 µmole p-nitrophenol per min at pH 4.6 at 50°C under the assay conditions.

Superoxide dismutase (SOD) assay

The fungal-related SOD activity was determined as described by Spychalla and Desborough, 1990. [26]. The enzyme extracts were obtained by more thorough disruption of the fungal grown fenugreek culture substrate using a waring blender at high speed for 2 min, so that the fungal mycelia was also completely disrupted before dialyzing as done for β-glucosidase studies. The enzyme reaction mixture containing 50 mM Na_2CO_3/$NaHCO_3$ buffer (pH 10.2), 0.1 mM EDTA, 0.015 mM ferricytochrome c and 0.05 mM xanthine was prepared. This mixture was used to blank the spectrophotometer at 550 nm. The assay was initiated by the addition of sufficient xanthine oxidase to produce a basal rate of ferricytochrome c reduction. One unit of SOD was defined as the amount of enzyme that inhibited the rate of ferricytochrome c reduction by 50 %

Antimicrobial assay

The anti-microbial efficacy of fenugreek sprout extracts to inhibit the growth of the bacteria *Helicobacter pylori* was studied. The bacteria was cultured on peptone agar plates containing 10 g peptone, 15 g granulated agar, 5 g sodium chloride, 5 g yeast extract, 5 g beef extract (Becton Dickinson and Co., Cockeysville, MD.) and 0.5 g of pyruvic acid in 1 L of water. They were maintained in a broth medium (same media mentioned above without agar) at 4°C. Stock cultures were grown at 37°C for 48 h prior to use. The plates were inoculated with 100 µL of overnight active culture and smeared for even bacterial growth.

Agar-diffusion test was done aseptically using sterile 1.2 cm diameter paper (susceptibility) disks purchased from Schleicher & Schuell, Inc., (Keene, NH 03431, USA). Round paper disks were sterilized and loaded with 50, 100, 150 and 200 µLs of the fungal-grown fenugreek extract. Saturated disks were then placed on top of the bacterial growth. Treated plates were inverted and immediately placed in an anaerobe jar using a Campylobacter microaerophilic gas generator with the catalyst in place (Microaerophilic Systems Envelops, Becton Dickinson & Company, Sparks, MD). Samples were incubated at 37°C for 18 h. The diameter of clear inhibition zone surrounding each disk was measured in centimeters.

α-Amylase inhibition disk assay

The α-amylase inhibition disk assay protocol developed by McCue and Shetty[27] was followed. A starch substrate media containing 5 g agar and 5 g starch in 500 mL distilled water was prepared, autoclaved and poured in petriplates. Sterile 1 cm diameter round paper disks was placed in the center of every petriplate with media. The amylase inhibition assay reaction mix contained 800 µL of the *Rhizopus oligosporus* bioprocessed fenugreek extract and 200 µL of the porcine pancreatic alpha-amylase solution (equivalent to 10 units in 20 mM sodium phosphate buffer pH 6.9). The samples were incubated at room temperature for 20 min. For the control 800 µL of the 20 mM sodium phosphate buffer pH 6.9 was used instead of the sample. Then 100 µL of the sample/control was loaded onto the sterile paper disk in the petriplates. Plates were sealed with parafilm and allowed to incubate for two days at room temperature. Five mL of iodine stain solution (5 mM iodine in 3% potassium iodide) was added to each plate and allowed to react for 15 min. Excess iodine stain was drained and the diameter of the clear zone was measured and used to calculate the amylase inhibitory activity. Results are reported as the Amylase Inhibition Percent, which is defined as the ratio of the amylase inhibition diameter of the (control- sample)/control x 100.

Statistical analysis

Experiments were run in triplicate and each SSB experiment was repeated 3 times. The average values of 9 measurements for each day with standard deviations are reported in the graphs. Statistical analysis was performed using MS-Excel software.

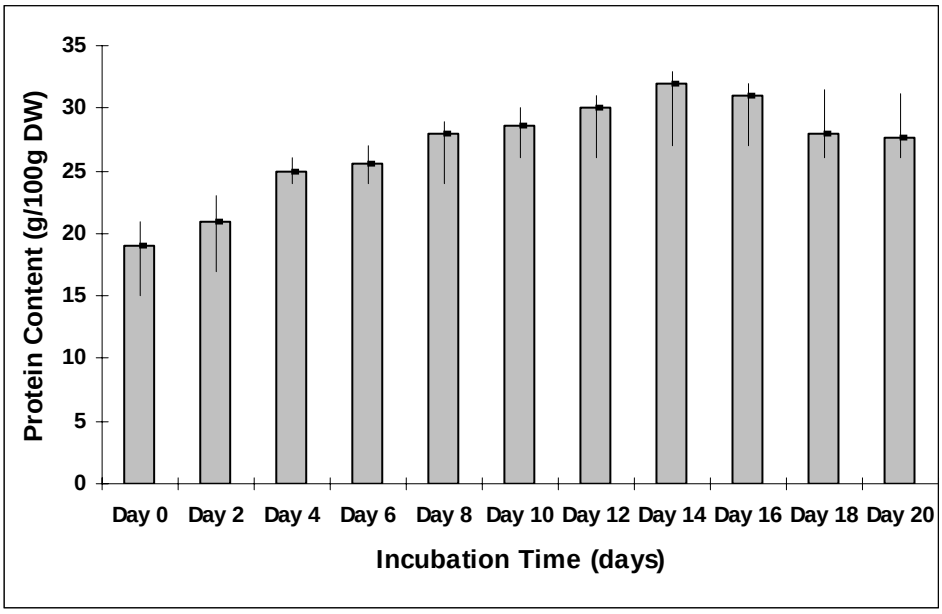

Figure 1. Protein content of fenugreek substrate during solid-state bioconversion by *Rhizopus oligosporus*

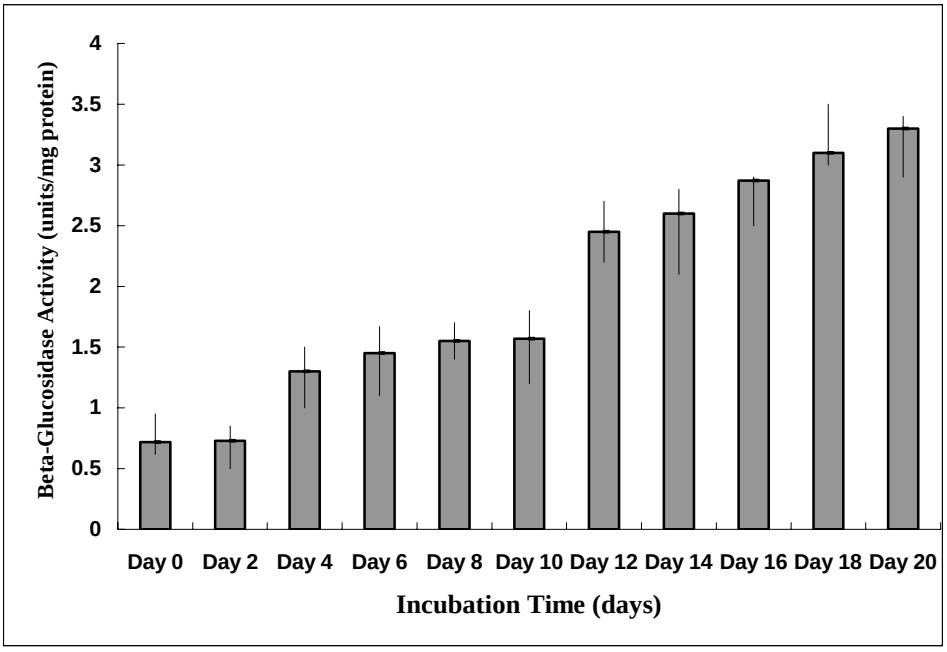

Figure 2. β-glucosidase activity in fenugreek substrate during solid-state bioconversion by *Rhizopus oligosporus*

RESULTS AND DISCUSSION

The growth of *Rhizopus oligosporus* fungus on the fenugreek substrate was rapid from day 4 of inoculation and completely colonized the entire substrate by day 8. The protein content of the inoculated fenugreek substrate, which is an indicator of the fungal growth, increased with incubation time (Fig 1). The protein content increased from 19 % during inoculation stage to a peak of 32 % on day 14 and subsequently maintained slightly lower levels throughout the rest of the growth period possibly due to nutrient depletion. Another indicator of the effective colonization of the substrate by the fungus is the β-glucosidase activity. Previous studies on fava bean seed powder bioprocessed by the same fungus showed a similar correlation.[17] The β-glucosidase activity was low initially (day 0-day 4) and then increased rapidly with growth (Fig 2). Higher levels were observed from day 4 to day 8, and then it further increased linearly from day 12 to day 20 of growth. Higher glucosidase activity also indicates the increased carbohydrate needs of the colonizing fungus.

Free phenolics usually exist in conjugate forms with one or more sugar residues bound to hydroxyl groups, or groups of compounds such as carboxylic and organic acids, amines and lipids. [28] Previous solid-substrate bioconversion of fava bean and soybean powders by the same fungus indicated that the fungal β-glucosidases mobilized the carbohydrate-bound or polymeric phenolics resulting in higher total phenolic content.[17,29] The change in the trend of total phenolics mobilized by *R. oligosporus*

growing on the fenugreek substrate is shown in Fig. 3. Fenugreek seeds are naturally very high in phenolics (14.3 mg/g DW), however the content increased to 23 mg/g DW on day 4 of growth. High levels were also observed during day 6, followed by slightly lower but stable levels throughout the rest of the growth period (14-17 mg/g DW). The increase in total phenolics on day 4 coincides with the initial increase in beta-glucosidase activity suggesting its possible involvement in phenolic mobilization and likely use of released sugars for energy. However unlike observations made in fava bean and cranberry pomace, solid-state bioconversion by the same fungus the higher activity did not correlate to higher total phenolics levels during late stages of growth (days 12-20) but only during early growth stages. [17,18] This suggests that other fungal enzymes may be involved in releasing phenolic aglycones and remobilizing from the fenugreek substrate during the different stages of bioprocessing. However in general the fungal enzymatic hydrolysis of these conjugated phenolics leads to increased free phenolic content, which can enhance the nutraceutical value of the fenugreek extracts.

In the present study, antioxidant activity of the bioprocessed fenugreek extracts was measured by the β-carotene bleaching method and DPPH free radical scavenging method. The β-carotene method estimates the

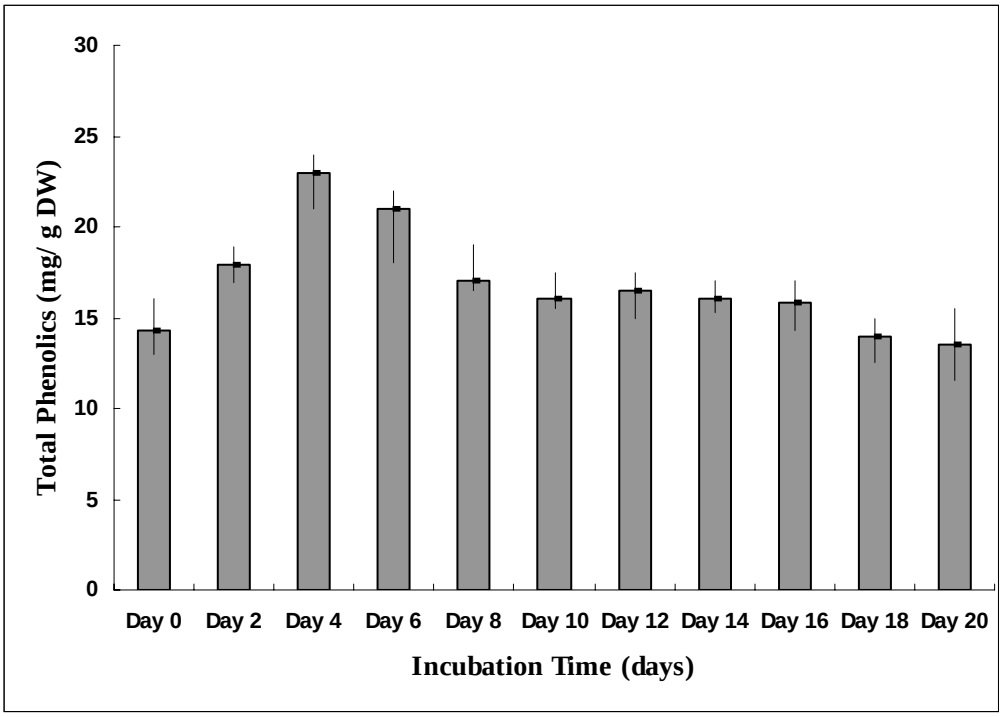

Figure 3. Total phenolic content of fenugreek substrate during solid-state bioconversion by *Rhizopus oligosporus*

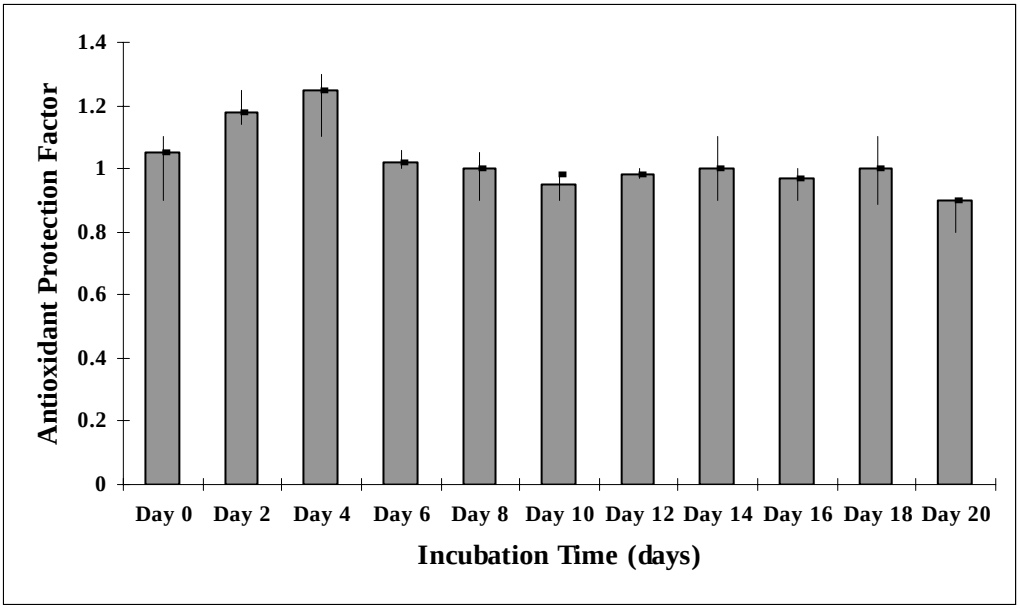

Figure 4. Antioxidant activity of fenugreek substrate during solid-state bioconversion by *Rhizopus oligosporus* as measured by β-carotene method

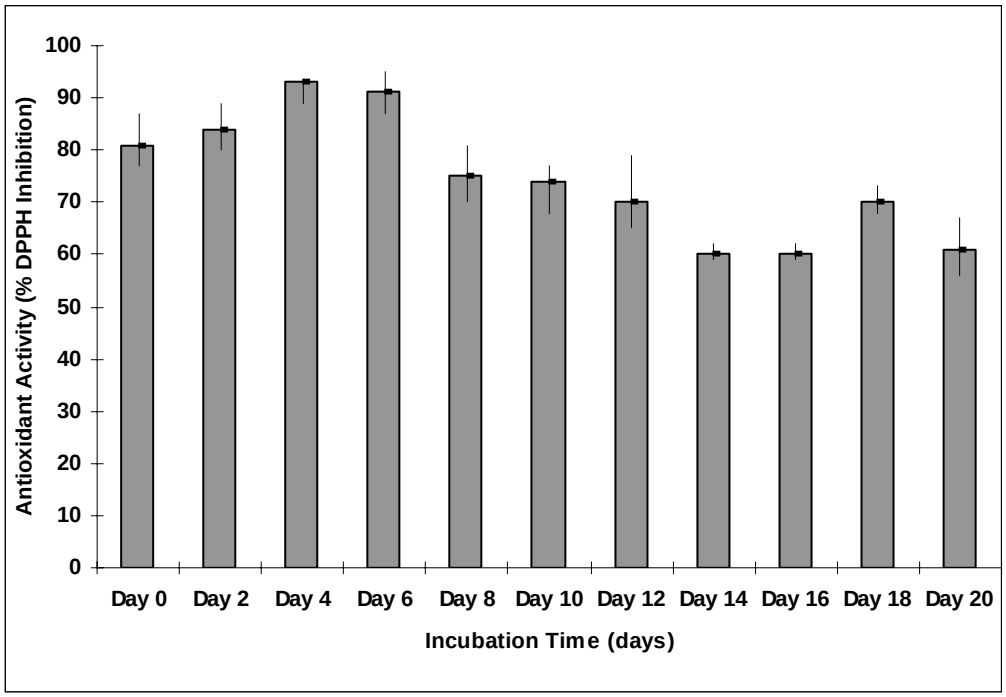

Figure 5. Antioxidant activity of fenugreek substrate solid-state bioconversion by *Rhizopus oligosporus* as measured by DPPH radical inhibition method

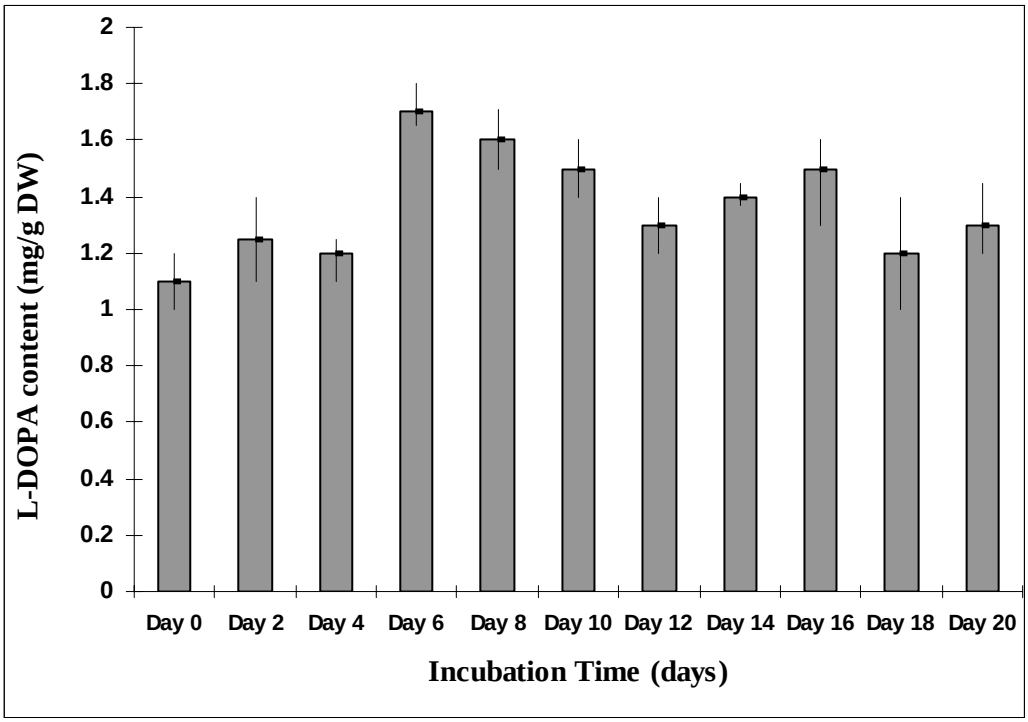

Figure 6. L-DOPA content of fenugreek substrate during solid-state bioconversion by *Rhizopus oligosporus*

ability of the bioprocessed fenugreek extract to function at a lipid–water interface to prevent H_2O_2 catalyzed β-carotene oxidation. The DPPH method estimates the ability of the extract to quench the DPPH free radical. The antioxidant activity measured by β-carotene assay (APF – Antioxidant protection factor) fluctuated during the bioconversion process (Fig 4). High APF was observed during early growth stage (day 2-4), followed by reduced but stable activity during later growth stage (day 6-day 20). The antioxidant activity of the bioprocessed fenugreek extracts as measured by the DPPH free radical scavenging method also showed high activity (93%) during early stages of growth (day 4–6) followed by lower levels during the later growth phase (Fig 5). A direct association between higher phenolic levels during early growth stage (days 4-6) and antioxidant activity was observed. This early stage activity may be linked to the mobilization of polymeric phenolic forms, since at this stage the APF

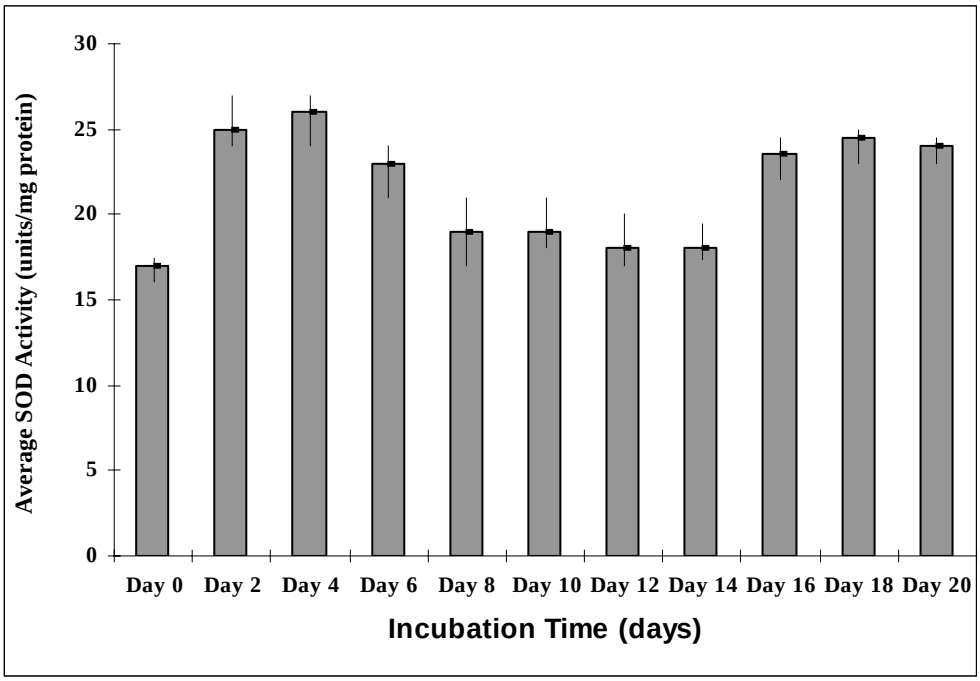

Figure 7. Fungus-linked SOD activity of fenugreek substrate during solid-state bioconversion by *Rhizopus oligosporus.*

linked activity was also high, which may be due to more hydrophobic phenolic forms. Since these phenolics appear to be effective quenchers of free radicals they possibly protect the fungus against elevated oxidative stress generated during early colonization of the fungus on the substrate. Reduction of phenoxyl radicals by reductants can recycle phenolic antioxidants, thus enhancing the antioxidant activity further. The antioxidant activity of phenolics is mainly because of their redox properties that allow them to act as reducing agents, hydrogen donors, singlet oxygen quenchers and metal chelators.[30]

The L-DOPA content of the fenugreek extract fluctuated during the course of bioconversion (Fig 6). In general higher levels were observed during days 6-10 (1.5-1.7 mg/g DW) and day 16 (1.5 mg/g DW). The early increase on day 6 may be the consequence of increased phenolic mobilization on day 4. Another factor could be that the increase in the protein content of the substrate during the bioconversion was making more tyrosine available for L-DOPA biosynthesis by the fungus. This increase could also be due to fungal tryrosine hydroxylase, which is the rate-limiting enzyme in the synthesis of L-DOPA from the tryrosine precursors.[31]

Superoxide dismutases (SOD) catalyze the dismutation of the superoxide radical to molecular O_2 and H_2O_2 thus preventing the oxidation of biological molecules, either by the radicals themselves, or by their derivatives.[32] As observed in the case of fava bean bioprocessing by the same fungus[17], in the present research the fungal superoxide dismutase activity initially was low on day 0 and increased significantly on day 2-6. Elevated SOD activity indicates the production of Reactive Oxygen Species during early bioconversion process that in turn triggered the elevated SOD expression in the fungus. Interestingly increased initial levels (days 2–6) coincide with higher antioxidant function when the likely oxidative stress of initial fungal colonization of the substrate was high. Slightly higher SOD activity on day 16-20 suggests another stress period of growth when there was a limitation of nutrients for fungal growth. This increase is directly proportional to an increased L-DOPA content suggesting the possible involvement of SOD during the L-DOPA mobilization from the substrate.

Figure 8. α-Amylase Inhibition disk assay. Left Petri-plate is control and right is fenugreek extract treated

The α-amylase inhibition percent (AIP) of the fenugreek seeds were naturally high (59%). Figure 8 shows α-amylase inhibition disk assay with control on the left with no inhibition and the fenugreek extract on the right with inhibition. All of the aqueous fenugreek extracts from the solid-state bioprocessing by *R. oligosporus* were found to possess significant α-amylase inhibitory activity. Bioprocessing substantially improved the AIP to 75 % on day 4 of incubation, followed by 73 % on day 6 (Fig. 9). The AIP reduced from day 8 onward maintaining lower levels throughout the rest of the incubation. The high AIP for day 4 coincides with higher levels of total phenolics and related antioxidant function. We suggest that the mobilized phenolic compounds with high antioxidant activity mobilized by *Rhizopus oligosporus* were contributing to the higher AIP. Other compounds present in the fungus

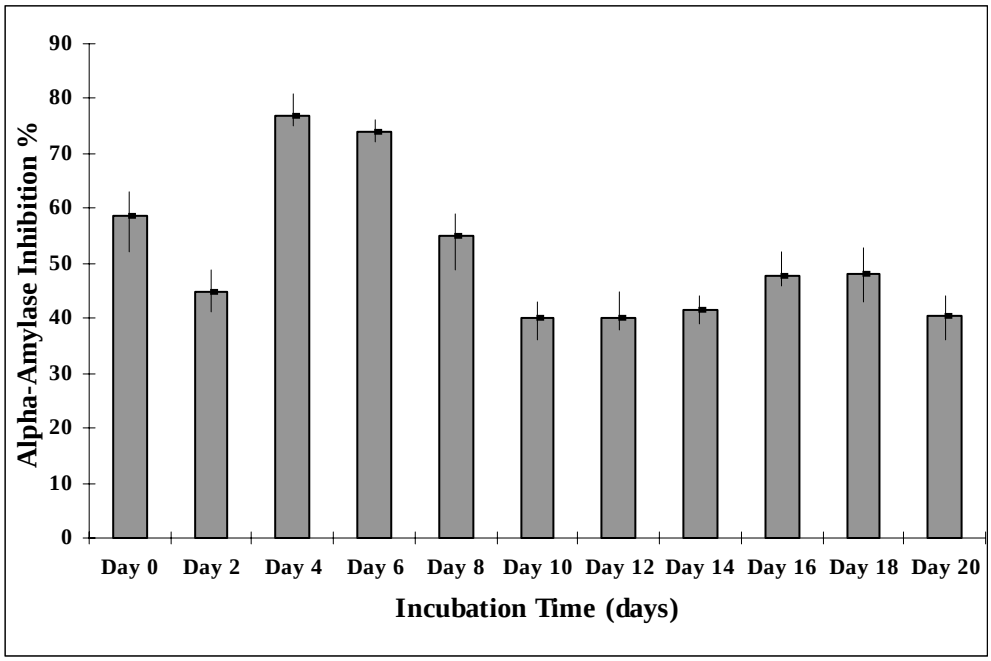

Figure 9. α-Amylase Inhibition Percent of fenugreek extracts during solid-state bioconversion by *Rhizopus oligosporus*

such as amino-sugars and carbohydrate analogues may also be responsible for improved AIP. Higher AIP may also be related to the structure of the simple phenolics and/or their modified forms present in the extracts during early incubation as observed in the case of pineapple.[19]

Phenolics are known to bind to the reactive sites of enzymes thus altering its catalytic activity. We suggest that the mechanism of inhibition of the glycolytic activity of α-amylase may occur through the direct blockage of the active center at several subsites of the enzyme as also suggested for other inhibitors.[9, 27] The α-amylase inhibitory factors present in the fenugreek extract probably interact with the active sites of the enzyme in a substrate-specific manner. Similar research with herbal extracts shows an association between antioxidant activity and higher AIP.[9, 27] The lower AIP observed in the late stages of incubation might be due to the breakdown of phenolic compounds with limitation of nutrients for fungal subsistence and acute oxidative stress as indicated by high SOD activity. There are numerous reports that indicate a connection between diabetes and impaired dopaminergic neurotransmission.[10, 11] Hence, amylase inhibition activity in a L-DOPA rich natural background would be a novel strategy to manage early stages of Type 2 diabetes linked to high glycemic index. Therefore day 6 could be the stage at which L-DOPA and potential synergistic phytochemicals could be extracted from the fungus colonized fenugreek substrate for such diet-based therapeutic applications.

We observed that fenugreek seeds naturally had *Helicobacter pylori* inhibition and hence investigated if the inhibition improved with SSB of fenugreek with *R. oligosporus.* Figure 10 shows the *H. pylori* disk assay, control (water only) exhibiting no inhibition is in the center, and disks loaded with varying concentrations of the fenugreek extract with inhibition zones are seen towards the periphery of the petriplate. In general extracts from all incubation times from day 0 to day 20 had some level of inhibition at all extract concentrations tested (50, 100, 150, 200 µLs). There was a direct correlation between the extract amount and inhibition diameter. The highest inhibition was seen with extracts from day 4, followed by day 6. Inhibition by extracts from days 8-12 was almost similar with reasonably higher inhibition (Fig. 11 shows inhibition for 100 and 200 µLs extracts only). Lower activity was observed during the later stages of incubation (days 16-20). Early stage extracts with high inhibition is associated with higher total phenolic content and associated antioxidant activity. This leads to the hypothesis that enhanced mobilization of carbohydrates by the fungus with perhaps phenolic release and polymerization as observed in the case of mung bean sprouts and cranberry contributed to high antioxidant activity and related *H. pylori* or α-amylase inhibitory activities.[18, 33]

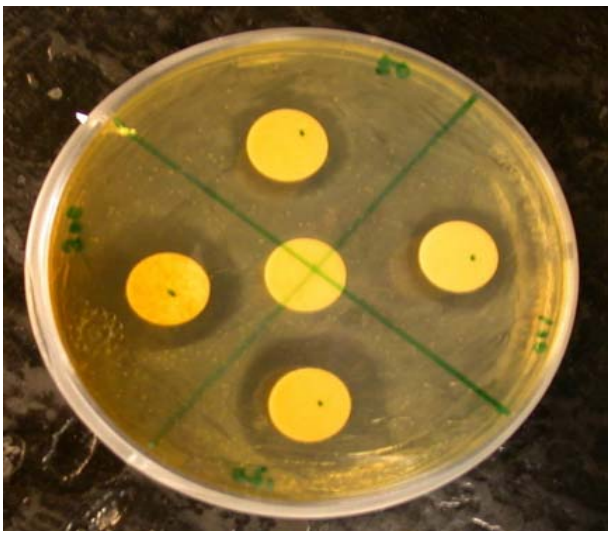

Figure 10. *Helicobacter pylori* inhibition disk assay. (Center disk represents control; Disk along periphery has increasing concentrations of fenugreek extract 50, 100, 150, 200 µLs starting from top disk in clockwise direction)

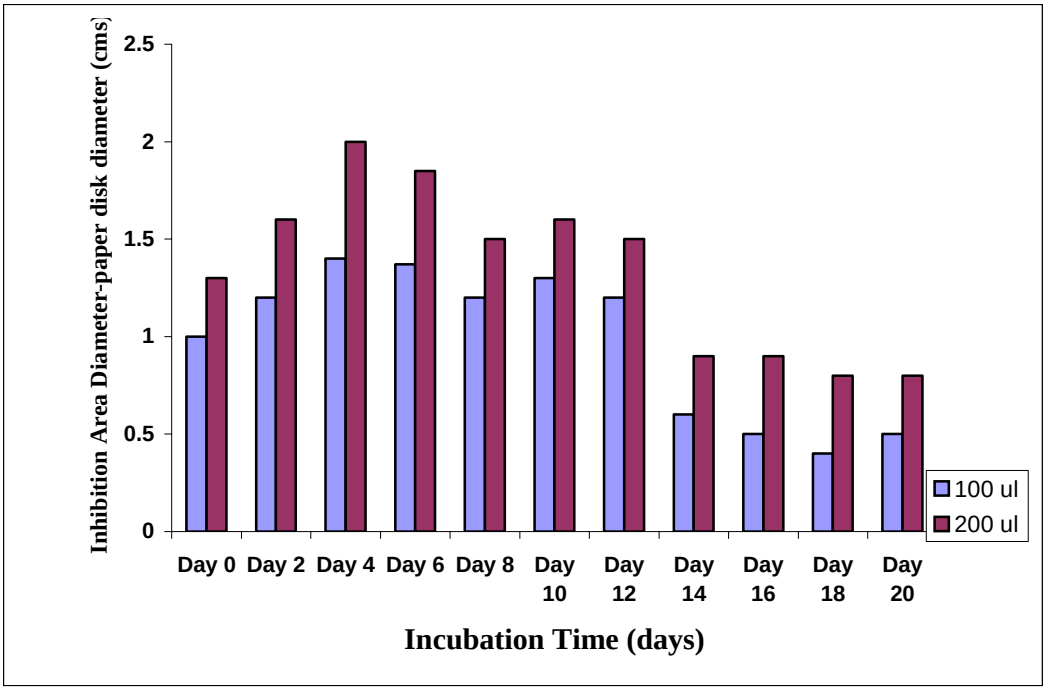

Figure 11. *Helicobacter pylori* Inhibition by fenugreek extracts during solid-state bioconversion by *Rhizopus oligosporus*.

We propose that the mechanism of antimicrobial activity by the phenolic containing extracts is by creating an acidic environment that causes the bacterial cell membrane to disrupt. Moreover plant phenolics and synthetic phenolics are known to have anti-bacterial properties by disrupting the membrane PMF (Proton Motive Force). The antimicrobial activity observed in fenugreek could also be due to presence of scopoletin, a coumarin derivative of coumaric acid and steroidal saponins.[34] Scopoletin is a lactonized phenolic, and has the potential to inhibit the electron transport chain in prokaryotes. There is also the possibility that the phenolics enriched fenugreek extracts were altering the urease activity of the bacteria. Urease is an extracellular enzyme and is also bound to the outer membrane of *H. pylori*, which provides the ideal alkaline microenvironment for its survival.[35] Other possible modes of action could be by lysis and leakage of intracellular constituents, perturbation of cell homeostasis, inhibition of enzymes, electron transport, and oxidative phosphorylation, interaction with macromolecules and effects on macromolecular biosynthetic processes by biocides as reported in other microorganisms.[36] HPLC analysis of solid-state bioprocessed pineapple extracts by the same fungus indicates that simple and biphenyl compounds contributed to high *H. pylori* inhibition[19] suggesting a similar mobilization mode in fenugreek.

IMPLICATIONS

SSB of the fenugreek substrate by *Rhizopus oligosporus* significantly enhanced the total phenolics and associated antioxidant activity resulting in improved α-amylase and *Helicobacter pylori* inhibition function. Thus this is an effective new strategy to significantly increase the natural alpha-amylase inhibitors associated with high phenolic antioxidants, which could potentially reduce the glycemic index thus helping in the management of carbohydrate metabolism disorders linked to Type 2 diabetes. From this study the early growth phase (days 4-6) when higher phenolics, antioxidant activity and higher L-DOPA content were mobilized, could be targeted for synergistic phytochemicals for functional food and therapeutic applications.

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REFERENCES

1. Raghuram TC, Sharma RD, Sivakumar B, Sahay BK. Effect of fenugreek seeds on intravenous glucose disposition in non-insulin dependent diabetic patients. Phytother Res. 1994;8:83-6.
2. Sharma RD, Sarkar A, Hazra DK, Mishra B, Singh JB, Sharma SK, Maheshwari BB, Maheshwari PK. Use of fenugreek seed powder in the management of non-insulin dependent Diabetes mellitus. Nutrition Research. 1996;16: 1331-39.
3. Broca C, Gross R, Petit P, Sauvaire Y, Manteghetti M, Tournier M, Masiello P, Gomis R, Ribes, G. 4-Hydroxyisoleucine: experimental evidence of its insulinotropic and antidiabetic properties Am J Physiol Endocrinol Metab. 1999;277:617-23.
4. Alcock NW, Crout ADHG, Gregorio MVM, Pike G, Samuel CJ. Stereochemistry of the 4-hydroxy-iso-leucine from *Trigonella foenum-graecum*. Phytochemistry. 1989; 28:1835-41.
5. Holman RR, Turner RC. Oral agents and insulin in the treatment of NIDDM. In: J. Pickup and G. Wiliams, Editors, Textbook of Diabetes, Blackwell, Oxford 1991;407-69.
6. Gallagher AM, Flatt PR, Duffy G, Abdel-Wahab WHA. The effects of traditional antidiabetic plants on in vitro glucose diffusion. Nutrition Research. 2003;23:413-24.
7. Marles RJ, Farnsworth NR. Antidiabetic plants and their active constituents, Phytomedicine. 1995;2:137-89.

8. Shani J, Goldschmied A, Joseph B, Ahronson Z, Sulma FG. Hypoglycemic effect of *Trigonella foenum graecum* and *Lupinus termis* (leguminosae) seeds and their major alkaloid in alloxan diabetic and normal rats. Archives of International Pharmacodynamics and Therapeutics. 1974; 210:27-36.
9. Payan, F. Structural basis for the inhibition of mammalian and insect alpha amylases by plant protein inhibitors. Biochimica et Biophysica Acta - Proteins & Proteomics. 2004;1696:171-80.
10. Murzi E, Contreras Q, Teneus L, Valecillos B, Parada MA, Parada MP, Hernandez L. Diabetes decreases limbic extracellular dopamine in rats. Neuroscience Letters. 1996;202:141-44.
11. Kono T, Takada M. Dopamine depletion in nigrostriatal neurons in the genetically diabetic rat. Brain Research. 1994;634:155-58.
12. Saller CF. Dopaminergic activity is reduced in diabetic rats Neuroscience Letters. 1984;49:301-06.
13. Maguire-Zeiss KA, Federoff HJ. Convergent pathobiologic model of Parkinson's disease. Ann. N. Y. Acad. Sci., 2003;99:152-66.
14. Randhir R, Lin YT, Shetty K. Phenolic, their antioxidant and antimicrobial activity in dark germinated fenugreek sprouts in response to peptide and phytochemical elicitors. Asia Pac J Clin Nutr. 2004;13:295-07.
15. Mitchell H, Megraud F. Epidemiology and diagnosis of *Helicobacter pylori* infection. Helicobacter, 2002; 7: 816.
16. Chun S, Vattem DA, Lin YT, Shetty K. Phenolic antioxidants from clonal oregano (*Origanum vulgare)* with antimicrobial activity against *Helicobacter pylori*. Process Biochemistry. 2005;40:809-16.
17. Randhir R, Vattem DA, Shetty K. Solid-state bioconversion of fava bean by *Rhizopus oligosporus* for enrichment of phenolic antioxidants and L-DOPA. Innovative Food Science & Emerging Technologies. 2004;5:235-44.
18. Vattem DA, Lin YT, Labbe RG, Shetty K. Antimicrobial activity against select food-borne pathogens by phenolic antioxidants enriched in cranberry pomace by solid-state bioprocessing using the food grade fungus *Rhizopus oligosporus*. Process Biochemistry. 2004;39:1939-46.
19. Correia RTP, McCue P, Magalhães MMA, Macêdo GR, Shetty K. Amylase and *Helicobacter pylori* inhibition by phenolic extracts of pineapple wastes bioprocessed by *Rhizopus oligosporus.* J Food Biochemistry. 2004;28: 404-19.
20. Nout MJR, Kiers JL. Tempe fermentation, innovation and functionality: update into the third millenium. J Appl Microbiol. 2005;98:789-05.
21. Shetty K, Curtis OF, Levin RE, Withowsky R, Ang W. Prevention of vitrification associated with in vitro shoot cultures of oregano (*Origanum vulgare*) by *Pseudomonas spp*. J Plant Physiol. 1995;147:447-51.
22. Hammerschmidt PA, Pratt DE. Phenolic antioxidants of dried soybean. J of Food Science. 1978;43:556-59.
23. Cervato G, Carabelli M, Gervasio S, Cittera A, Cazzola R, Cestaro B. Antioxidant properties of Oregano (*Origanum vulgare*) leaf extracts. J Food Biochemistry. 2000;24: 453-65.
24. Bradford M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Annals of Biochemistry. 1976;72:248-54.
25. Hang YD, Woodams EE. Apple pomace: a potential substrate for production of small β-glucosidase by *Aspergillus foetidus*. Food Science and Technology. 1994;27: 587-89.
26. Spychalla JP, Desborough SL. Superoxide dismutase, catalase and alpha tocopherol content of stored potato tubers. Plant Physiology. 1990;94:1214-18.
27. McCue P, Shetty K. Inhibitory effects of rosmarinic acid extracts on porcine pancreatic amylase in vitro. Asia Pac J Clin Nutr. 2004;13:101-06.
28. Bravo, L. Polyphenols: Chemistry, Dietary Sources, Metabolism, and Nutritional Significance. Nutritional Reviews. 1998;56:317-333.
29. McCue P, Horii A, Shetty K. Solid –state bioconversion of phenolic antioxidants from defatted soybean powders by *Rhizopus oligosporus*: Role of carbohydrate –cleaving enzymes. J Food Biochemistry. 2003;27:501-14.
30. Rice-Evans C, Miller N, Paganga G. Antioxidant properties of phenolic compounds. Trends in Plant Science. 1997;2:152-59.
31. Borges RC, Geddes T, Watson JT, Kuhn DM. Dopamine biosynthesis is regulated by S-glutathionylation. Potential mechanism of tyrosine hydroxylase inhibition during oxidative stress. J of Biological Chemistry. 2002;277: 48295-302.
32. Fridovich I. Superoxide radical and superoxide dismutases. Annual Review of Biochemistry, 1995;64:97-112.
33. Randhir R, Lin YT, Shetty K. Stimulation of phenolics, antioxidant and antimicrobial activities in dark germinated mung bean sprouts in response to peptide and phytochemical elicitors Process Biochemistry. 2004;39:637-46.
34. Basch E, Ulbricht C, Kuo G, Szapary P, Smith M. Therapeutic applications of fenugreek. Altern Med Rev. 2003; 8:20-7.
35. Hawtin, P.R., Stacey, A.R. and Newell, D.G., 1995. Investigation of the structure and localization of the urease of *Helicobacter pylori* using monoclonal antibodies. J Gen Microbiol. 1995;136:1995-00.
36. Eklund T, Nes IF. Effects of biocides on DNA, RNA and protein synthesis. Soc Appl Bacteriol Tech Ser. 1991;27: 225-34.

Original Article

Improved α-amylase and *Helicobacter pylori* inhibition by fenugreek extracts derived via solid-state bioconversion using *Rhizopus oligosporus*

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葫蘆巴籽萃取物經過 *Rhizopus oligosporus* 的固態形式生物轉換改善 α-澱粉酶及幽門螺旋桿菌抑制作用

本研究透過以真菌為基礎的固態形式生物轉換（SSB）系統，使葫蘆巴籽(*Trigonella foenum graceum*)基質酚類抗氧化劑與 L-DOPA 強化。這個方法採用食物等級的黴菌 *Rhizopus oligosporus*，選擇的原因為它已經被指出對其他種籽及食物基質具改善健康相關功能之效益。在亞洲地區被運用在食品加工已經有長久歷史。基質中蛋白質含量及 β-葡萄糖苷活性反應真菌生長情形，他們的量隨著培養時間增加及增加的酚類的量增加而增加。同時也指出他們可能參與酚的流動。抗氧化活性是採用 β-胡蘿蔔漂白及 DPPH 自由基去除方法分析，兩者都顯示在早期生長期(第 4-6 天)有高活性，後期生長期(第 8-20 天)活性則下降。早期生長期(第 4-6 天)較高的酚類含量與抗氧化活性直接的相關，指出其與聚合過程及疏水性酚類形式的關聯性。葫蘆巴籽萃取物的 L-DOPA 含量在生物轉換過程中起伏，在第 6-10 天量較高(1.5-1.7 mg/g DW)。SSB 過程顯著促進了豬隻 α-澱粉酶體外試驗抑制活性，在第 4 天時達 75%，此與萃取物高量的總酚類及相關抗氧化物活性有關。α-澱粉酶抑制活性的高峰也伴隨著高的 L-DOPA 含量。這些結果對糖尿病飲食管理有其意義；相同的生物轉換階段的幽門螺旋桿菌抑制活性對潰瘍管理也有意義。

關鍵字：葫蘆巴籽、α-澱粉酶抑制作用、第二型糖尿病、固態形式生物轉換(SSB)、***Rhizopus oligosporus***、β-葡萄糖苷、抗氧化物活性、L-DOPA(levo-二羥基苯丙酮胺酸)、超氧化歧化酶、抗氧化物保護因子、幽門螺旋桿菌。