

Safety assays and nutritional values of mycoprotein produced by *Fusarium venenatum* IR372C from date waste as substrate

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Abstract

BACKGROUND: Nutritional and environmental benefits of mycoprotein verify its beneficial role on the health of humankind in the next decades. Agro-industrial wastes can be used as cheap substrates to decrease the total cost of product. However, fungi may produce toxins or lead to allergic reactions in consumers. Therefore, the study of the safety and nutritional aspects of this product are very important.

RESULTS: *Fusarium venenatum* IR372C was cultured on date wastes and ammonium salts in submerged fermentation. The safety and nutritional issues of produced mycoprotein were investigated including allergy tests and analyses of toxins, as well as existence of toxin genes, and content of heavy metals, metals, amino acids and fatty acids. The results showed that fumonisin genes in *F. venenatum* IR372C remain without any gene expression during 1 week fermentation. Zearalenone and deoxynivalenol cannot be detected in the fermentation medium after 3 weeks. Prick tests on 30 volunteers demonstrated no sensitivities to mycoprotein. The content of lead was $658 \mu\text{g kg}^{-1}$ as the highest heavy metal followed by arsenic, cadmium and mercury at 161, 30.57 and $0 \mu\text{g kg}^{-1}$, respectively. Produced mycoprotein includes essential amino acids at appropriate contents and the ratio of unsaturated to saturated fatty acid was nearly 2:1. Also, calcium, iron, magnesium and zinc were found in mycoprotein, which could have health beneficial impacts on consumers.

CONCLUSION: This study has provided information on safety aspects of mycoprotein production by *F. venenatum* IR372C from date wastes. However, further studies with focus on long-term clinical benefits of diets containing mycoprotein are necessary.
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Keywords: allergic reaction; date syrups; fungal toxins; mycoprotein; nutritional values; submerged fermentation

INTRODUCTION

In recent decades, increased demand on food and changes in climates due to greenhouse gas effects have led to a trend toward alternative sources for the production of protein such as microbial production, e.g. mycoprotein with numerous benefits including time, energy and cost savings as well as environmental sustainability. Recently, all characteristics of mycoprotein as meat alternatives including sensory, environmental, economic as well as marketing, health and safety aspects have been reviewed.¹ Production of mycoprotein does not result in changes in climates, increases in greenhouse gas emissions, degradations of lands or pollution of freshwater resources.²

The cost benefit aspect of mycoprotein depends on the substrate, while it should be food-grade and contain carbohydrates and ingredients required for the appropriate microbial growth.³ The use of agro-industrial wastes will solve several problems with one single action: conversion of wastes to value added food.⁴ Production of mycoprotein has been reported from various waste

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materials such as industrial pea processing byproduct,⁴ cactus pear biomass,⁵ potato waste,⁶ grass silage fiber,⁷ sugar waste⁸ and oil.⁹

Date fruit is an agro-industrial product in tropical climate, e.g. south Iran and other countries of the Persian Gulf region. Sub-standard dates are low-cost and available substrates with no inhibitors for microbial growth. Based on the literature, date waste has been reported as a substrate for the production of xanthan,¹⁰ single cell oil,¹¹ baker's yeast biomass,⁵ lactic acid,¹² alkaline protease¹³ and cultivation of mushrooms as well as other microbial metabolites.

Naturally, a wide range of microorganisms are capable of producing single cell protein.^{3,14,15} Ascomycetes such as *Neurospora* spp., *Monascus* spp. and *Fusarium venenatum* are generally regarded as safe microorganisms.¹⁶ To date, a few studies have been carried out on submerged fermentation due to some benefits of culture homogeneity, reproducible attribute and a better mass-heat transfer in comparison to solid-state fermentation.^{14,17}

In previous studies, optimal processes for the production of mycoprotein by *F. venenatum* IR372C from date wastes as substrates in surface¹⁵ and submerged¹⁰ culture have been reported. The main purpose of the earlier studies included screening of novel microorganisms and substrates¹⁸ as well as characterization of the products^{3,15,18} and optimization of the fermentation processes.^{3,15} To the best of the authors' knowledge, there is a gap in the research for a comprehensive investigation of safety aspects. Although filamentous fungi are choices for mycoprotein production, they have the potential to produce toxins or lead to the incidence of allergies in consumers.¹⁹

The major aim of this study was the investigation of all safety aspects of mycoprotein production including existence of toxin encoding genes using polymerase chain reaction (PCR), toxin contents using high-performance liquid chromatography (HPLC) by ultraviolet (UV) (for deoxynivalenol), flame ionization detector (FLD) (for zearalenone) and mass spectrometry (MS) (for fumonisin), and allergic reactions using prick test. Furthermore, nutritional aspects including the profile of amino acids (AAs) and fatty acids were investigated by HPLC and gas chromatography-flame ionization detector (GC-FID), respectively. Also, heavy and

nutritional metals were measured by inductively coupled plasma-mass spectrometry (ICP-MS).

MATERIAL AND METHODS

Date wastes as substrates

Substandard dates (low-quality and damaged fruits) were collected from farms and date packaging factories and transferred to the laboratory. To remove plant debris, dirt and sand particles, a cleaning step was carried out by blowing air on the dates and brushing them. Cleaning was performed carefully to avoid further damage or cross contaminations to fruit inner parts. For maximum extraction, dates were transferred to containers and sufficient volumes of water (water/date ratio of 3:1) were added. Temperature and time of blender was set at 70 °C for 30 min. The blending was carried out at a constant rate and in covered containers to minimize evaporation of water. Then, mixtures were filtered to achieve clear solutions. Ethylene bromide as fungicide was added to the solutions to inhibit growth of fungi. This chemical was used only to maximize the extraction process to remove fungi interference. After the study on gene expression and allergic reactions in a 3-L lab scale bioreactor, there was no need for its usage in pilot scale (20-L) production and sensory evaluation due to safety concerns for residue. Solutions with Brix of 12 to 15 were heated at 55–60 °C under vacuum and concentrated to Brix of 70 to prolong their shelf life.

Date chemical analyses

Chemical compositions of the date syrups were analyzed based on the approved methods by the Association of Official Analytical Chemists (AOAC).²⁰ Analyses included measurement of moisture content by using vacuum oven drying at 80 °C; ash content by heating at 550 °C. Also, fat and protein contents were determined by Soxhlet extraction (with hexane) and micro-Kjeldahl method (with nitrogen to protein factor of 6.25). Total carbohydrate content was also measured by subtracting the total percent values of other analyses from 100. Total soluble solids (Brix) were calculated using a refractometer (Carl Zeiss, Oberkochen, Germany).²⁰

Microorganisms, media, inoculums and culture conditions

Fusarium venenatum IR372C had been purchased for previous studies^{10,15} from the Iranian Research Institute of Plant Protection (IRIPP), Tehran, Iran. The strain was cultured on agar-solidified Vogel slants and incubated at 25 °C for 48 h and then stored at 4 °C according to Hosseini et al.³ For the preparation of the fungal suspension, 5 mL of sterilized deionized water were added to slant and a concentration of 10⁵ colony-forming units (CFU) mL⁻¹ was prepared using microbial count slide. Inocula were prepared by adding fungal suspensions in 250-mL conical flasks containing 200 mL of Vogel's media and incubating in a shaker incubator (Jaltajhiz, Iran) at 180 rpm for 48 h at 25 °C.

Fermentation

Submerged fermentation was carried out using a 3-L stirred-tank bioreactor (Winpact, Taoyuan, Taiwan). Vogel media, containing date syrup instead of glucose, were used as fermentation substrate. The optimal condition was adjusted based on a protocol by Reihani and Khosravi-Darani,¹⁵ with mild modification (Table 1). After a heat shocking step (72 °C, 10 min) at the end of the process (to simulate ribonuclease and reduce RNA content of mycoprotein¹⁵), biomass was separated using a kitchen sieve and dried using a freeze dryer (Martin Christ, Osterode am Harz,

Table 1 Composition of fermentation culture (modified Vogel) and condition of operation for mycoprotein production by *Fusarium venenatum* IR372C on date waste

Component	(g L ⁻¹)
Na ₃ citrate	2.5 g
KH ₂ PO ₄ (anhydrous)	5.0 g
NH ₄ NO ₃ (anhydrous)	2.0 g
MgSO ₄ ·7H ₂ O	0.2 g
CaCl ₂ ·2H ₂ O	0.1 g
Distilled water	1.0 L
Trace element solution	0.1 mL
Biotin solution (50 g mL ⁻¹)	50 µL
Date syrup	20
Operation	
Aeration	1 (vvm ⁻¹)
Stirring	400 rpm
Temperature	28 °C
Time	72 h
pH	5.6

ALPHA 2–4 LDplus, Germany). The protein content was assessed using Lowry method with partial modification according to Slocombe *et al.*²¹

Food safety considerations

The control of safety factors in dates (especially low-quality dates) and fungal biomasses (which are used as ingredients in food formulations) is important to approve or reject the final products. Relatively, natural contaminants, including fungal toxins (mycotoxins), allergens and heavy metals such as lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg), must be investigated. In the present study, no microbial contaminations were reported due to sterilization of date syrups and other ingredients before processing.

Mycotoxin analysis

Zearalenone and deoxynivalenol

Incubation

Preparation of the incubating media was carried out as previously described for inoculums with sterilized date syrups used instead of glucose syrups (similar to real fermenting cultures in fermenters). Inoculums were prepared in 5% v/v, containing 10^5 CFU mL⁻¹. Flasks (three replicates) were incubated at 27 ± 2 °C for 3 weeks in the dark using a shaker incubator at 180 rpm to induce microbial toxin production.

Extraction and cleanup

At the end of the incubation period, the flasks content and date wastes (for controlling safety of substrates and their roles in mycoprotein safety) were dried at 40 ± 2 °C for 24 h using a hot-air oven. Preparation and analysis steps were carried out according to Rauová.²² Extracts were further purified using filtration through 0.45-µm Whatman Puradisc 25AS filters (Whatman, Clifton, NJ, USA) and aliquots were used for HPLC analyses. To validate method, the toxin standard was spiked into control samples. Method validation was investigated by carrying out recovery studies using fungi media samples spiked with the evaluated mycotoxins at three concentrations of 500, 1000 and 2000 ppb. Each experiment was conducted in three replicates. Intermediate precision was assessed by analyzing spiked samples at the same concentrations. Repeatability and intermediate precision are obtained by the relative standard deviation (RSD %), while trueness is expressed by recovery values.

Materials and reagents

Reference chemicals substances of zearalenone (ZON) (98% purity) were purchased from Sigma-Aldrich (Steinheim, Germany) and deoxynivalenol (DON) (97% purity) was from Sigma-Aldrich (Madrid, Spain). All chemicals included were analytical or HPLC grade. Organic solvents of HPLC grade were purchased from Waters (Milford, MA, USA). Other chemicals of analytical grade were purchased from Merck (Darmstadt, Germany). Stock standard solutions of ZON and DON with concentrations of

1.0 mg mL⁻¹ were respectively prepared in methanol and acetonitrile and stored at -18 °C until use. Calibration curves were prepared using eight various ZON and DON concentrations of 25 to 250 ng mL⁻¹ ($R^2 > 0.982$) for the standards and 50 to 2500 ng mL⁻¹ for the samples ($R^2 > 0.999$), respectively

HPLC apparatus and analysis

In this study, Waters 2695 system (Waters, Milford, MA, USA) with UV Detector 2489 and Fluorescence Detector 2475 was used. Furthermore, reverse-phase Agilent Eclipse XDB C18 Column (4.6 mm × 50 mm, 1.8 µm) protected with a guard (Agilent Technologies, Santa Clara, CA, USA) was used for the study. The ZON was measured using fluorescence detection and DON using UV detector at 218 nm. Identification of mycotoxins was carried out using a simple comparison of retention times, eventually through absorption spectra in the spotted wavelength range. Each sample was analyzed in duplicate.

Fumonisin

Identification of fumonisin gene using polymerase chain reaction DNA extraction was carried out according to a previous study.²³ Two pairs of primer were used based on the GenBank database sequences for *fum1* to detect fumonisin-producing microorganisms and for *rDNA* to identify fungi genus. Primer sequences and PCR conditions are listed in Table 2. The amplification mixture (Sigma, India) consisted of 18.25 µL of double distilled water (ddH₂O), 1 µL of genomic DNA, 1 µL of magnesium chloride at 50 mmol L⁻¹, 2.5 µL of PCR buffer, 0.75 µL of each dNTP, 0.5 µL of Taq polymerase and 0.5 µL of each primer. The PCR cycling conditions included an initial denaturation at 94 °C for 4 min, followed by 30 cycles at 94 °C for 60 s, 58 °C for 60 s and 72 °C for 90 s with a final extension at 72 °C for 8 min (MWG PCR machine; Biotech, Ebersberg, Germany). Agarose gel of 1.2% w/v (12 g agarose in 1000 mL Tris-acetate-EDTA) electrophoresis was used for the PCR products.²³

Liquid chromatography–mass spectrometry (LC–MS)

Liquid chromatography–mass spectrometry (LC–MS) as a fairly sensitive analytical technique was used for fumonisin detection.²⁴ After incubation for 1 to 3 weeks, the extraction process was carried out based on European Standard EN 114352:2004 with mild modification according to Dohnal *et al.*²⁵ Briefly, 25 mL of the extraction solution (methanol/acetonitrile/water of ratio 1:1:2 v/v/v) were added to 5 g of the filtrate culture in flasks and mixed well for 2 min. Then, flasks were centrifuged (Sigma 3 K30, Sigma-Aldrich, Germany) at $1400 \times g$ for 5 min. Supernatants were filtered using 0.45-µm Whatman Puradisc 25AS filters (Whatman) prior to HPLC–MS analysis. The HPLC system included vacuum degasser, auto-sampler and binary pump (Agilent Series 1200, Agilent Technologies). The system was equipped with a reversed-phase rapid resolution C₁₈ analytical column of 50 mm × 4.6 mm and 1.8-µm particle size (RR Zorbax Eclipse

Table 2 Primer sequences and their conditions for toxin analysis by PCR in *Fusarium venenatum* IR372C

Primer name	Gene target	Amplicon size (bp)	Melting temperature (°C)	Sequence (5'–3')	Reference
ITS 1	rDNA	550	58	CAACTC CCAACCCCTGTGA	22
ITS 2				GCGACGATTACCAGTAACGA	
Fum 1-4583 f	fum 1	698	59	GGGTTCACAGGCAGATCGTAC	23
Fum 1-5283 r				GAACCTCACATCCTTTGGGCC	

XDB-C₁₈, Agilent Technologies). Mobile phases A and B included water with 0.1% of formic acid and acetonitrile, respectively. The HPLC system was connected to a triple quadrupole mass spectrometer (Agilent 6410 Triple-Quad LC/MS, Agilent Technologies) equipped with an electrospray interface operating in positive-ion mode. The operation parameters included capillary voltage of 5000 V; nebulizer gas of 50 psig; gas flow of 12 L min⁻¹; gas temperature of 325 °C and inject volume of 10 µL. Nitrogen served as nebulized and collision gas. Specific MRM transitions, fragmentor voltages and collision energies were optimized according to a previous study.²⁶

Heavy metals

Sample preparation. All the chemical reagents were of analytical grade. The digestion step was carried out according to Hseu with partial modification.²⁷ Dried fungal biomass (0.1 g) and date syrup (1 g) were separately added to 50-mL digestion tubes and 5 mL of concentrated nitric acid were added to each tube. Samples were heated at 80 °C for 45 min; then, the temperature was increased to 100 °C and samples were boiled for at least 2 to 3 h until clear solutions were achieved. Solutions were filtered using No. 42 Whatman and 0.45-µm Millipore filter papers. Filtrates were transferred to 25-mL volumetric flasks and volumes were adjusted using distilled water. Contents of metals were assessed using ICP-MS (Agilent 7700x, Agilent Technologies, Tokyo, Japan). The dilution coefficient was calculated in the final result.

Instruments and analyses. In this study, an ICP-MS instrument (Agilent 7700x, Agilent Technologies, Tokyo, Japan) equipped with a quadrupole mass spectrometer was used for the assessment of metals in solutions of date syrups and fungal biomasses. Operating conditions for the ICP-MS instrument were adjusted as previously described by Itoh *et al.*²⁸ Standard solutions for the calibration curves in ICP-MS were prepared by diluting commercial multiple-element standard stock solutions (100 µg mL⁻¹ each) (Inorganic Ventures IV-STOCK-8SPEX, Metuchen, NJ, USA). Furthermore, the internal standard correction was carried out using beryllium (Be), indium (In), and thallium (Tl) as internal standard elements to correct matrix effects due to major elements. Limit of detection (LOD) for the metals analyzed by ICP-MS included 1 µg kg⁻¹ (except for Hg and Pb, LOD of 5 µg kg⁻¹).

Allergy skin tests

Preparation of extracts. Protein was extracted from the fungal biomasses (mycoprotein) as described previously by Assarehzadegan *et al.*²⁹ In brief, 0.17 g of the *F. venenatum* IR372C dried biomass was mixed with 10 mL of 0.01 mol L⁻¹ phosphate-buffered saline (pH 7.4) by continuous stirring at 4 °C for 18 h. The extract was centrifuged at 16 000 × *g* and the supernatant dialyzed against 10 mmol L⁻¹ of phosphate buffer. The extract was filtered through a 0.22-µm membrane under sterile conditions and then freeze dried.

Volunteers and skin prick tests. Skin prick tests were carried out by an experienced nurse under a physician's supervision. In this method, one drop of the fungal biomass extract was transferred onto the patients' inner forearm and irritation of the epidermis was carried out using prick method. After 15 min, the mean diameter of the wheal reaction was measured and compared with negative (50% glycerin) and positive (histamine diphosphate, 10 mg mL⁻¹) controls. Skin prick test with a wheal

diameter > 3 mm was reported as positive, compared to negative and positive controls.²⁹

Nutritional values

Fatty acid profile

Folch method was used to extract lipids from the samples (chloroform/methanol 2:1 v/v).³⁰ Fatty acid methyl esters were methylated following a method described by the European Official Methods of Analysis with partial modification.^{31,32} Fatty acid methyl esters were assessed using GC (Agilent 6890, Agilent Technologies, USA) fitted with a FLD and capillary column (30 m per 0.25 mm ID, film thickness of 0.25 µm) (Thermo TR-5, Thermo-Fisher Scientific, Waltham, MA, USA) operated with helium as the carrier gas (0.2 mL min⁻¹) according to Hashempour-Baltork *et al.*³³ Identification of the fatty acid profile was carried out by the comparison of sample chromatograms with reference methyl ester ones (Sigma Aldrich, St Louis, MO, USA).

Amino acid profile

Briefly, 1 g of dried fungal biomass was hydrolyzed with 1 N hydrochloric acid (50 mL) using a boiling water bath according to Czauderna *et al.*³⁴ The sample was centrifuged at 10 000 × *g* to recover hydrolysate. Then, 100 µL of the hydrolysate were injected into HPLC instrument (Waters, Milford, MA, USA) equipped with Zorbax Eclipse-AAA Column (4.6 mm × 150 mm, 5 µm) (Agilent Technologies, USA) at 40 °C and fluorescence detector. Moreover, 40 mmol L⁻¹ sodium dihydrogen phosphate (NaH₂PO₄) was used as mobile phase A and acetonitrile/methanol/water at a ratio of 45:45:10 v/v/v was used as mobile phase B.

Minerals

Method for the assessment of nutritional compounds was similar to the method described for heavy metals.^{27,28}

Statistical analysis

Descriptive data were reported as mean ± SD (standard deviation). Three replicates were used for each sample of each test. Data were analyzed statistically using SPSS Statistical Software v.17 (SPSS Inc., Chicago, IL, USA).

RESULT AND DISCUSSION

Date syrup production

Date syrups from the present method contained nearly 70% (w/w) of total solids (fructose of 36.1%, glucose of 29.02%, ash of 2.4%, protein of 1.58% and fat of 0.08) with a moisture content of 30% (pH 4.7). The nutritional values were similar to those from previous studies.^{3,15} From each 1 kg of dates, approximately 650 g of 70°-Brix date syrup were achieved.

Fungal biomass production

The fermentation process resulted in a high-protein biomass with a high degree of fibrosity. The high-protein biomass or mycoprotein produced by this method included a texture similar to bread dough or chicken breast (by observation and no instrumental measurement). The biomass typically included 55% (w/w) of protein in a total dried biomass. This value was approximately equal to or even higher than values reported in the literature,^{2,3,35} indicating an optimized procedure.

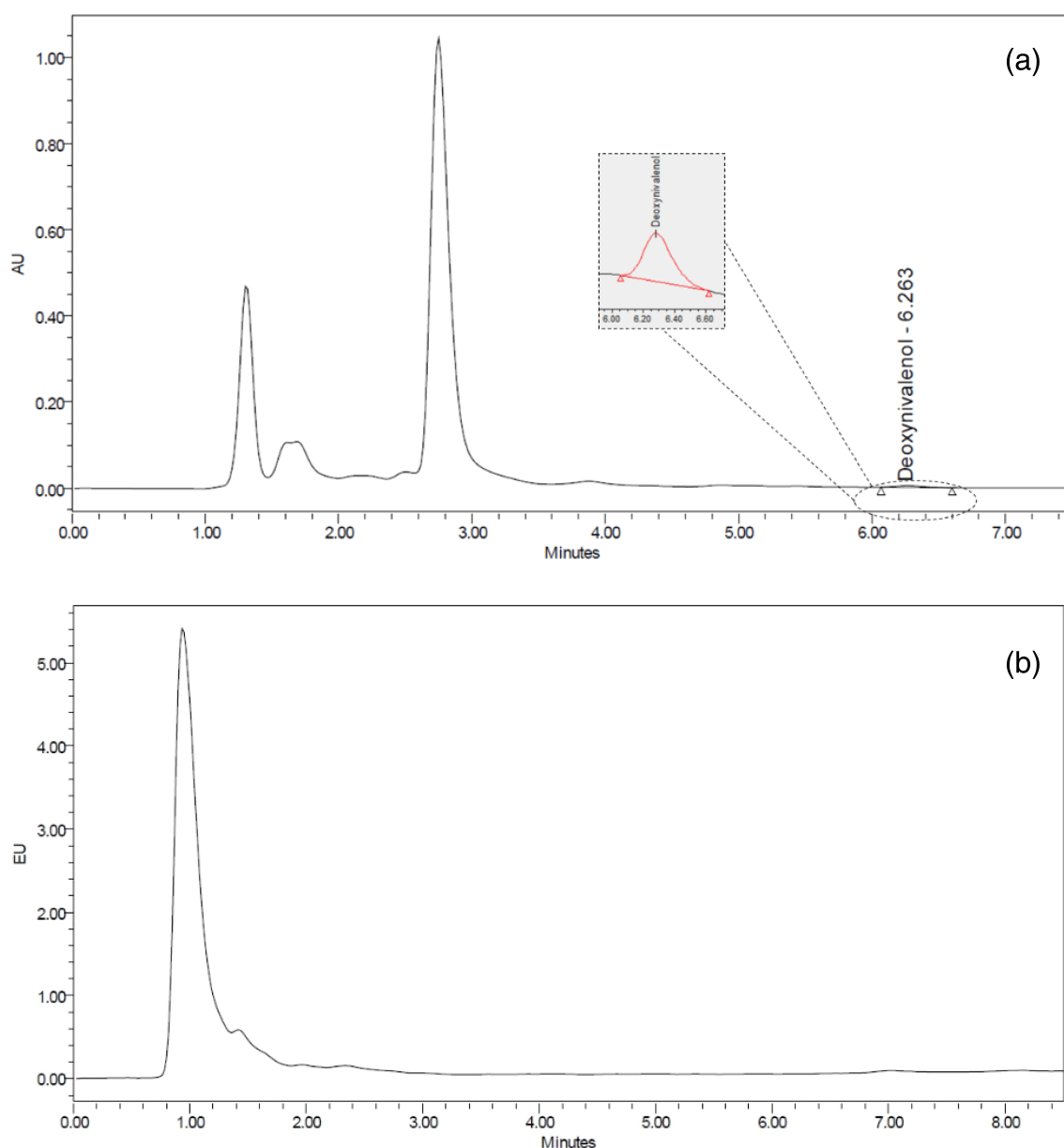


Figure 1 HPLC chromatograms for presence of DON (a) and ZON (b) in raw date syrup. DON = $1620 \mu\text{g L}^{-1}$, ZON = not detected (LOD = $0.3 \mu\text{g L}^{-1}$). The retention time of DON and ZON were about 6.3 and 5.7 min, respectively.

Safety aspects

Toxin analysis for DON and ZON

Toxin analysis results are presented in Fig. 1. The LOD and limit of quantification (LOQ) included 0.3 and 1 ng g^{-1} , respectively. The LOD and LOQ of the method were reported using spiking samples at 1 ng g^{-1} . Retention times of the ZON and DON included 5.7 and 6.3 min, respectively. Results showed $1620 \mu\text{g kg}^{-1}$ of DON for raw date syrup with no ZON. No DON or ZON was reported in *F. venenatum* IR372C culture after 3 weeks of incubation. Although 72 h is an appropriate time period to complete the fermentation process, incubation continued for 3 weeks to ensure that there was no possible production of toxins. However, results verified no DON or ZON in *F. venenatum* IR372C after 3 weeks. DON can inhibit protein synthesis and ZON are estrogenic metabolites, which have potential threats to animals and humans.^{36,37} In

addition, results showed toxins that initially existed in raw date syrup were not detected in the final medium culture. This could be an important observation which could be interpreted as decontamination potential of fungus. Until further studies are conducted we can only speculate this observation by possible metabolizing of toxin in a single strain culture of fungus. However, some similar results are reported by other researchers,^{38–40} which showed the potential ability of bacteria and fungi for bioremoval of toxins. These results are significantly important and can help resolve concerns about use of wastes as substrates for mycoprotein production by *F. venenatum* IR372C. Previous reports on toxicology tests of the *F. venenatum* A 3/5 showed no toxin productions by this strain.⁴¹ Analysis of mycotoxin in Quorn strain of *F. venenatum* NRRL 25416 by the commercial company demonstrated that four types of trichothecenes were formed at low

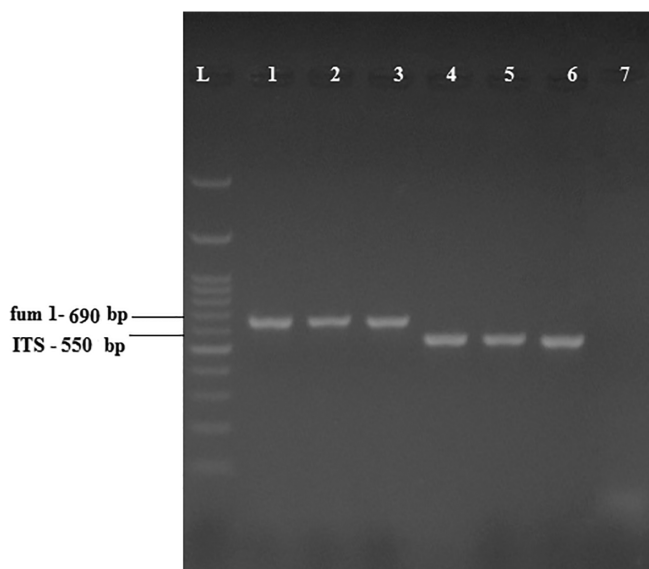


Figure 2 Agarose 1.2% gel showing the PCR products: Singleplex PCR assays with primers Fum 1F/ Fum 1R and internal transcribed spacer (ITS) were used to determine Fumonisin (690 bp) and rDNA (550 bp), respectively; Lane L: 100 bp DNA ladder; 1: fumonisin-positive control (*Fusarium proliferatum* MTCC 286); 2: fumonisin-positive control (*Fusarium moniliforme* ITCC 3362); 3: sample strain (*Fusarium venenatum* IR372C); 4–6: represent positive and sample strains for ITS; 7: negative control.

levels. Although, none of these trichothecenes were reported in Quorn final products at $0.5 \mu\text{g g}^{-1}$ LOD,⁴² which demonstrated that no toxins were produced during cultivation or removed during further processing. Since *F. venenatum* NRRL 25416 has the potential to produce trichothecenes, a careful monitoring of the samples seems necessary for every batch to ensure the safety of mycoprotein products.

Identification of fumonisin gene using polymerase chain reaction

In the current study, specificity of the primers was assessed using single-PCR of the genomic DNA from standard fungal isolates (Fig. 2). Concentrations of the targeting genes were adjusted to provide similar intensities of the amplified products (Fig. 2). As shown in Fig. 2, *F. proliferatum* MTCC 286, *F. moniliforme* ITCC 3362 and the sample strain included rDNA and fumonisin-

Table 4 Volunteer characteristics for prick test

Sex (%)	Age range	Reaction to skin prick test
Female (40%)	20–60	Negative ^a
Male (40%)	20–60	Negative
Child and teenager (20%)	7–15	Negative

^aNegative means none of the volunteers indicate any sensitivity to biomass extract but indicate sensitivity to histamine as positive control.

encoding genes with 550 and 690 bp, respectively. Sensitivity of the PCR might be affected by various biological components such as fat, phenolic and polysaccharide compounds, which could decrease purity of the extracted DNA.⁴³ In the present study, the highest efficacy of the assay was achieved using an efficient DNA extraction method and optimized PCR condition. Based on the results, *F. venenatum* IR372C included encoding genes of fumonisin and hence could potentially be dangerous for mycoprotein production for human use. Thus, further analyses for toxin gene expressions are required to find appropriate solutions. According to Dilkin et al.,⁴⁴ moisture content, temperature and time are the most important factors influencing fumonisin production in food. The highest yield of fumonisin synthesis is reported at a moisture content of 34%, temperature of 25 °C and culture time of 60 days.⁴⁴

LC–MS for fumonisin qualification

The three samples that include (i) control (with no fungi), (ii) culture after 1 week and (iii) culture after 3 weeks incubation were analyzed by LC–MS. By comparing the results, the third week sample (iii), demonstrated fumonisin peaks with retention times of 1.8 to 2 min, while no peaks were detected in the control (i) and the first-week sample (ii). As stated previously, mycoprotein production needs a fermentation time of 72 h and then the toxin production stops or decreases after a heating shock, food processing or refrigerator storage. The results indicated that *F. venenatum* IR372C was able to produce fumonisin but not in a short time (< 1 week). This finding was similar to a finding by Dilkin et al.,⁴⁴ who reported that the lowest time for fumonisin production included nearly 10 days of incubation.

Table 3 Heavy metals in date syrup and biomass *Fusarium venenatum* IR372C on date waste

Heavy metals			Maximum limit (DW) ⁴⁶			Nutritional metals			
Compound	Date syrup	Dried mycoprotein	Meat and chicken	Soybean	Unit	Compound	Date syrup	Dried mycoprotein	Unit
Pb	63.29 ± 0.02 ^a	658.99 ± 0.08	400	—	$\mu\text{g kg}^{-1}$	Ca	0.505 ± 0.20	1.017 ± 0.20	mg g^{-1}
As	76.61 ± 0.05	161.84 ± 0.09	—	—	$\mu\text{g kg}^{-1}$	Fe	3.334 ± 0.50	54.427 ± 0.45	$\mu\text{g g}^{-1}$
Hg	ND ^b	ND	—	—	$\mu\text{g kg}^{-1}$	Mg	0.233 ± 0.30	1.271 ± 0.40	mg g^{-1}
Cd	3.57 ± 0.08	30.57 ± 0.01	200	200	$\mu\text{g kg}^{-1}$	K	2.246 ± 0.90	18.376 ± 0.98	mg g^{-1}
						Na	0.477 ± 0.02	5.969 ± 0.05	mg g^{-1}
						Zn	1.945 ± 0.50	60.942 ± 0.20	$\mu\text{g g}^{-1}$
						Se	0.074 ± 0.04	0.161 ± 0.02	$\mu\text{g g}^{-1}$

^aMean ± standard deviation.

^bND, not detected. ICP–MS LOD = $1 \mu\text{g kg}^{-1}$ for all metals except Hg and Pb (LOD = $5 \mu\text{g kg}^{-1}$).

Table 5 Amino and fatty acid composition of mycoprotein of *Fusarium venenatum* IR372C

Amino acid	Content (% g per 100 g protein)	Fatty acid	Content (% w/w)
L-Alanine	5.920 ± 0.99 ^a	<i>Saturated fatty acid</i>	
L-Arginine	6.920 ± 0.187	Myristic (C14:0)	0.52 ± 0.000
Aspartic acid	4.200 ± 0.030	Palmitic (C16:0)	21.24 ± 0.29
L-Cystine	1.201 ± 0.05	Margaric (C17:0)	0.53 ± 0.001
L-Glutamic	12.902 ± 0.12	Stearic (C18:0)	8.99 ± 0.161
Glycine	4.150 ± 0.353	Arashidic (C20:0)	0.519 ± 0.048
L-Histidine	3.200 ± 0.85	Gadoleic (C20:0)	0.194 ± 0.003
L-Isolucine	4.850 ± 0.363	Behenic (C22:0)	0.421 ± 0.034
L-Leucine	6.750 ± 0.353	Linoceric (C24:0)	0.739 ± 0.69
		Total	33.153
L-Lysine	7.500 ± 0.661	<i>Monounsaturated fatty acid</i>	
L-Methionine	2.900 ± 0.807	Heptadec-9-enoic (C17:1)	0.19 ± 0.019
		Oleic (C18:1)	18.21 ± 0.365
		Erucic (C22:1)	0.142 ± 0.017
		Total	18.54
		<i>Polyunsaturated fatty acid</i>	
L-Serine	5.350 ± 0.253	Linoleic (C18:2)	29.4 ± 0.353
L-Theronine	4.750 ± 0.707	Linelaideic (C18:2 t)	0.056 ± 0.002
L-Tyrosine	4.350 ± 0.907	α-Linolenic (C18:3)	18.180 ± 0.748
L-Valine	5.090 ± 0.707	γ-Linolenic (C18:3)	0.328 ± 0.002
		Total	47.96

aMean ± standard deviation.

Heavy metals

Adverse pollutions of heavy metals such as Pb, As, Cd and Hg are apparent and also increase in the human environment and food by different ways. Humans are unavoidably exposed to these heavy metals which have harmful effects to health, especially in childhood.⁴⁵ Table 3 describes primary concentrations of heavy metals in date syrups and fungal biomasses after fermentation using ICP-MS analysis. As mycoprotein is still a new alternative source of protein, there is no regulation for permissible limits of heavy metals in it. However, standards of chicken, meat and soy protein were used in the current study due to similarities in uses and characteristics. Based on the results, the mean contents of Pb, As, Hg and Cd in dried mycoprotein included 658.99, 161.84, 0 and 30.57 µg kg⁻¹, respectively. Permitted limits for these metals have been set by the European Countries⁴⁶ for various red meats and chicken meats [Pb of 400 µg kg⁻¹ dry weight (DW) and Cd of 200 µg kg⁻¹ DW; Hg and As not set] and soybean (Cd of 200 µg kg⁻¹ DW).⁴⁶ In the present study, contents of Pb was higher than the allowable limit and Cd was less than the standard limits. These permitted limits have been set based on a meat consumption rate of 43 kg per capita yearly;⁴⁷ however, consumption of meats is actually more than consumption of mycoprotein due to the meat sensory characteristics. Daily consumption of mycoprotein products is relatively low, thus, it cannot be considered as a serious source of heavy metal uptake, but according to the expanding trends of Quorn production in recent years, the amount of mycoprotein consumption may increase in the near future. According to our results it was indicated that no heavy metals were found in date syrups but were detectable in mycoprotein products, therefore, it seems that the source of contamination was the water supply.

Allergy skin tests

An allergic reaction is one of the major food safety issues. Consumption of novel dietary protein, including mycoprotein, may result in allergic reactions.² Food allergy is defined as 'an immune system-mediated adverse reaction to food proteins'. Food can cause allergic reactions but more than 90% of the food allergies are caused by cow's milk, soybean, egg, peanut, wheat, fish and shellfish.⁴⁸ In this study, 30 volunteers were tested for their reactions to fungal biomass extracts using the skin prick test. The skin prick test is a reliable method to diagnose immunoglobulin E (IgE) mediated allergic reactions in patients, including anaphylaxis, asthma, urticaria, atopic eczema, drug allergy and suspected or novel food. Furthermore, the test is minimally invasive, low-cost and reproducible and immediately responds.⁴⁹ The characteristics of the volunteers are shown in Table 4. In this study, volunteers were equally selected in sex and age. Results indicated that none of the volunteers had an allergic reaction to the testing extracts. According to Finnigan *et al.*,² published and unpublished evidence have shown that intolerance reactions or allergies to Quorn products are rare and very low, compared to common allergenic food. In contrast, Jacobson and DePorter,⁵⁰ reported that 1752 allergic and gastrointestinal reactions were linked to consumption of Quorn products. Three hundred and twelve people showed anaphylaxis and urticaria after 4 h and 1692 people demonstrated emesis and diarrhea within 8 h of consumption. Furthermore, some people experienced both adverse reactions. In the present study, mycoprotein extracts included no symptoms in the 30 prick tests.

Nutritional aspects

Fatty acids

Fatty acid composition of fungal biomasses is presented in Table 5. Comparison of the contents with previously reported

contents highly confirmed the results, especially in the ratio of unsaturated and saturated fatty acid of 2:1.¹⁵ In 2009, Hosseini et al.³ reported a ratio of 3.2–3.5 to 1 for unsaturated and saturated fatty acids. Indeed, consumption of high quantities of unsaturated fatty acids provides health benefits in heart diseases. The polyunsaturated and saturated fatty acids ratio of beefs is typically near 0.1 and this ratio decreases with increases in fatness of the meats.⁵¹ This ratio is reached to 1.44 in mycoprotein. Chicken fat contains 30% of saturated fatty acids, 45% of monounsaturated fatty acids and 21% of polyunsaturated fatty acids,⁵² which are similar to mycoprotein fatty acid profiles from Table 5.

Amino acids

The AA profile of fungal biomasses almost included essential amino acids (EAAs). This finding is similar to that from studies, which reported the presence of EAA in single cell protein from *F. venenatum*.^{3,15} This similarity suggests that the AA profile of mycoprotein is not affected by the substrates or fermentation methods. Three categories of food deliver nearly 80.9% of the total daily protein (meat and meat products, 38.9%; grain products, 23.9%; and milk and dairy products, 18.1%).⁵³ The important point of protein nutritional aspects includes net protein utilization, protein efficiency ratios, EAA, digestibility and biological values. Recently, Hashempour-Baltork et al.¹ compared protein qualities of mycoprotein and meats. Both sources were close from nutritional index viewpoints.

Nutritional minerals

Elements such as sodium (Na), potassium (K), magnesium (Mg) and calcium (Ca) and trace elements such as iron (Fe), zinc (Zn), copper (Cu), manganese (Mn) and cobalt (Co) are essential for human health. Well-balanced diets provide adequate quantities of the necessary elements for the body. Inadequate intakes of essential minerals can lead to serious nutritional deficiencies, including impaired growth, lethargy, reduced exercise performance and attention span and deficiencies in synthesis of the organic matrices of bones.⁵⁴ Dietary reference intakes (DRIs) in the United States are reference values of nutrient intakes to use for healthy diets. In this study, quantities of minerals in mycoprotein were analyzed and listed in Table 3. Contents of nutritional minerals are similar to those in most recommended daily food, e.g. milk, meat and fruit. According to the literature, meat included Fe, Zn, Mg, Na, K and Ca with 2.7, 4.1, 22, 63, 350 and 0.5 mg kg⁻¹, respectively.⁵⁵

CONCLUSION

Production of mycoprotein by *F. venenatum* IR372C from date wastes as carbon sources is an ecofriendly process. Toxin analysis showed no toxic concerns in the products (mycoprotein). To decrease possibility of heavy metal toxicities, further attentions are necessary for water supplies and raw materials. Allergy tests indicated no reactions to mycoprotein extracts. Amino and fatty acid profiles showed EAA and a 2:1 ratio of unsaturated/saturated fatty acids. The AA profile of mycoprotein was not affected by the substrates or fermentation methods. The primary toxin contents of raw date syrups were decontaminated by the fungi. Therefore, the investigation on decontamination potential of this fungus will be the aim of our next study in the near future.

More investigations on the possibility of synthesis of other mycotoxins including Fusarin C, Diacetoxyscirpenol Culmorin,

Enniatin B, S and other secondary metabolites of *F. venenatum* strains is recommended for future studies.

Daily consumption of mycoprotein may increase in the near future, so, regulation and standards should be released by governmental organizations for permissible limits for heavy metal uptake.

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