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Erythritol (Noncaloric) Production from Fermented Defatted Egyptian Soybean Using *Trichoderma reesei*

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Abstract: Erythritolis used as healthy noncaloric, sweetener to drinks. The present study threw light on the possibility to produce erythritol (low calorie) from soybean manufacturing wastes using fungal strains namely *Tricoderma reesei* F-417, *Penicillium funiculosum* F-229, *Tricoderma koningi* F-27 and *Asperigillus fumigatus* F-55. Volatile compounds were extracted from fermented defatted soy meal (FDSM). Extracts were subjected to: identification of volatiles compounds by GC/ MS and determination of antioxidant activity using DPPH method and ß -carotene-linoleate scavenging assay. Results revealed thatthe fungus *Penicillium funiculosum* F-229, *Tricoderma koningi* F-27 and *Asperigillus fumigatus* F-55 can not produce erythritol while the microbial *T. reesei* F-417 canproduce glycerol, arabinitol and ribitol which converts to erythritol. A total of 17 volatile components were identified in defattened soybean products after incubation with *Trichoderma reesei* F-417 for 3 days at 30°C On the other hand, the obtained results showed that defatted soybean by *T. reesei* F-417 has high antioxidant compounds and therapeutic effect.

Key words: Fermented Soybean · Erythritol GC-MS · Antioxidan

INTRODUCTION

Functional drinks in the market represent a large variety, including energy drinks and sport drinks and different types of tea (green, black and oolong and herbal) juice drinks and milk drinks. These products become successful if they are of faithful health benefits and good taste.

Erythritol is sugar alcoholic natural sweetness represent about 60 -70% of the sweetness of sucrose. It is used today by many beverage manufacturers as a beneficial healthy noncaloric, sweetener and is also an antioxidant [1].

Low-calorie foods are considered of great importance for obese patients and type 2 diabetes. Erythritol is noncaloric (≤ 0.2 kcal/g) so it is useful as a sweetener in most weight loss programs. The human body does not contain enzymes to digest and break down, where as erythritol excreted in the urine unchanged and remains about 10% of the erythritol be digested by fermentation by the colonic microflora produced short-chain fatty acids, which are metabolized in the liver [2].

Free radicals such as hydroxyl group (OH•) consists, stimulating the metal elements such as iron and copper that destroy the cell walls and nucleic acids, leading to cell death [3].

Some researchers have proved that erythritol is instrumental to get rid of these free radicals [4] and did not produce evidence of toxicity [5].

Erythritol consists naturally in various fruits and fermented foods can be produced by the process of microbial fermentation using mutant strains of *Aureobasidium sp., Trichonsporonoides sp. and Pseudozymatsukubaensis* [1]. It occurs frequently in fermented food including wines, beers and processed vegetables such as soy sauce and oriental miso bean paste [6-8].

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The aim of the present work was to study the possibility of simultaneous production of erythritol (few calories) and to identify the volatile components of waste soybean manufacturing products by fermentation with different fungal strains *Tricoderma. reesei* F-417, *Penicillium funiculosum F-229, Tricoderma koningiF-27* and *Asperigillus fumigatusF-55*.

MATERIALS AND METHODS

Chemicals and Reagents: 2, 2'-diphenyl-1-picrylhydrazyl (DPPH) and β-carotene were purchased from Sigma Chemical (St. Louis, MO). Liquid chromatography grade of all solvents were obtained from Merck (Darmstadt, Germany).

Microorganisms and Culture Conditions: Tricoderma reesei F-417, *Penicillium funiculosum F-229, Tricoderma koningi F-27* and *Asperigillus fumigatusF-55* were obtained from Microbial Chemistry Lab. National Research center, Dokki, Cairo.Egypt and maintained on potato dextrose agar slants at 30°C for 72 hrs. The spore suspensions were prepared by adding 10 ml of sterilized water to slant cultures and the surface was gently rubbed with a sterilized wire loop. The fermentation was carried out in 250 ml Erlenmeyer flasks containing 5g of defatted (5 % of fat) soybean flour moistened to50 % (v/w) with distilled water. One milliliter of spore suspension (10^6 spores) was used as inoculum. The cultures were incubated at 30°C for 3 days by solid state fermentation.

Extraction of Volatile Compounds: Volatile compounds were extracted from fermented defatted soy meal (FDSM)] [9, 10]. Fermented defatted (5 % fat) soy meal was ground individually into powder. The ground soy part (5.00 g) was weighed into a 25mL test tube and extracted using 15 mL ethanol. the solvent layer was separated from the solid residue by centrifuging at 2000 ×g for 10 minutes. The clear supernatant was transferred to a clean test tube. Then the solid residue was extracted with another 15 mL of ethanol. The separated ethanol layers were combined and dried using a vacuum evaporator at less than 50°C. The dried soy bean extract was weighed and stored at -20°C until samples were subjected to:

- Identification of volatiles compounds by GC/ MS
- Determination of antioxidant activity (Free Radical Scavenging Capability) using the DPPH assay and β-carotene-linoleate scavenging assay.

Gas Chromatographic-mass Spectrometric Analysis (GC/ MS): The analysis was carried out by using a coupled gas chromatography Hewlett-Packard model (5890) / mass spectrometery Hewlett-Packard-MS (5970). The ionization voltage was 70 eV, mass range m/z 39-400 a.m.u. The oven temperature was maintained initially at 50°C for 5 min. and then programmed from 50 to 250°C at a rate of 4°C/min. Helium was used as the carrier gas, at flow rate of 1.1 ml/min. The injector and detector temperatures were 220 and 250°C, respectively. The isolated peaks were identified by matching with data from the library of mass spectra (National Institute of Standard and Technology) and compared with those of authentic compounds and published data. The quantitative determination was carried out based on peak area integration. Interpretation on mass spectrum GC-MS was conducted using the database of (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

Determination of Antioxidant Activity

β-Carotene-Linoleate Scavenging Assay: The antioxidant activity of the extracts were evaluated using β-carotene-linoleate model system. 0.1 mg of β-carotene in 0.2 mL of chloroform, 10 mg of linoleic acid and 100 mg of Tween-20 were mixed. The solvent was removed at 40°C under vacuum and the resulting mixture was diluted with 10 mL of water and was mixed well. To this mixture, 20 mL of oxygenated water was added. Four milliliter aliquots mixtures were pipetted into different test tubes containing 200 µL of each extract (50, 100, 200 and 400 µg/ml) and TBHQ (50, 100, 200and 400 µg/ml) in ethanol. TBHQ was used for comparative purposes. A control containing 200 µL of ethanol and 4 mL of the above emulsion was prepared. The tubes were placed at 50°C in a water bath and the absorbance at 470 nm was taken at zero time (t= 0). The absorbance was continued to be measured until the colour of β-carotene disappeared in the control tubes (t =60 min) at an interval of 15 min. A mixture prepared as mentioned above without β-carotene served as blank. All determinations were carried out in triplicate. The antioxidant activity (AA) of the extracts was evaluated in terms of bleaching of the β-carotene using the following formula,[11].

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

Where: A_B: absorption of blank sample (t=0 min).
A_A: absorption of sample solution (t=60 min).

Radical Scavenging Activity Using DPPH Assay: Antioxidant activity was also determined by DPPH assay using spectrophotometer at 517 nm.[12]. Each extract of different concentrations (50, 100, 200 and 400 µg/ml, respectively) and TBHQ (50, 100, 200and 400 µg/ml) were taken in different test tubes. Four milliliter of 0.1 mM methanol solution of DPPH was added to these tubes and shaken vigorously. The tubes were allowed to stand at room temperature for 30 min. The control was prepared as the same without any extract and MeOH. The changes in the absorbance of the prepared samples were measured at 517 nm. Radical scavenging activity was estimated as the inhibition percentage and was calculated using the following formula,

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \text{ X100}$$

Where: A_B: absorption of blank sample (t=0 min),
A_A: absorption of sample solution (t=30 min).

Statistical Analysis: The results are reported as Mean ± Standard deviation (S.D.) for at least three times experiments. Statistical differences were analyzed by one way ANOVA test.

RESULTS AND DISCUSSION

Volatile Components in Fermented Soybean: The volatile compounds in various fermented soybean products, such as Japanese miso and natto, Chinese sufu and Thai thua nao, have been studied extensively [13-18]. Nearly 100 different volatile compounds representing a variety of chemical classes were identified. There is wide variation in the volatile components of fermented soybean products, studies have shown that the most frequently present compounds include esters (ethyl 2-methyl butyrate, ethyl hexanoate), acids (acetic acid, 2/3- methyl butanoic acid), pyrazines and phenolic compounds. The volatile component profiles of products vary with the micro-flora involved, as well as by the processing conditions (e.g. fermentation, drying, brining, or ageing) [13, 16, 18]. The volatiles in fermented soybean were extraction and then analysed by GC–MS. Tables 1, 2, 3 and 4 list the identified volatile components in each of microorganisms. A total of 17 volatile components in table 1, including 2 alkanes, 2 esters, 5 alcohols, 5 acids, 2 ketones and 1 aromatic compound, were identified. Amongst the volatiles; alcohols, acids and ketone were the largest groups. Glycerol (1,2,3 propanetriol), n-Hexadecanoic acid, oleic acid and 9,12- octadecadienoic acid were major volatiles. A total of 13,14 and 11 volatile components in Tables 2, 3 and 4 respectively were identified. Amongst the volatiles, esters were the largest groups in Tables 2,3 and 4. 4-[Phenylethynyl] acetophenone in tables 2,3 and 2,3-Bis endo diacetyl-7-antiformyl-norbornene in Table 4 were major volatiles. All the detected esters were previously found in various fermented soybean foods [13, 15, 19-22]. A number of high molecular weight fatty acid were detected and have also been found in Chinese sufu [13], miso[15] and other Korean fermented soybean pastes [19-22]. Most esters have characteristic odours. Alcohols were also reported in soy sauce [23], miso [15]and Korean doenjang [20-22] as important contributors of flavour. Ketones can be

Table 1: GC-MSanalysis of volatile chemical compounds of fermented soy bean by *T. reesei F.417*

Chemichal Groups	Identified compounds	Area %	Rt
Acids	n-Hexadecanoic acid	9.36	36.12
	Oleic Acid	9.48	39.45
	9,12-Octadecadienoic acid	14.84	39.37
	9-Octadecenoic acid	2.17	39.82
	Pterin-6-carboxylic acid	1.35	31.05
Alcohols	1,2,3Propanetriol	11.20	18.90
	Butane,1,2,3,4-Tetraol	1.50	29.04
	Ribitol	1.39	34.87
	D-L-Arabinitol	1.98	35.87
	Erythritol	29.90	29.72
Alkanes	2-Trifluoroacetoxydodecane	2.01	10.16
	3-Dimethylsilyloxytridecane	1.54	11.84
Aromatic compound	1-Methoxy-2-tertbuty l-4-propyl-6-methylbenzene	1.56	26.38
Esters	Methyl-7,8-Dideutero-7-nonenoate.	0.57	8.45
	Ethyl -isoallocholate	1.38	52.21
Ketones	Pantolactone	5.47	13.39
	3,3-Dideuterio-endo -6-hydroxy-9-oxa-bicyclo-nonan-2-one	2.29	24.72

Table 2: GC-MSanalysis of volatile chemical compounds of fermented soy bean by *Penicillium funiculosum F-229*

Chemichal Groups	Identified compounds	Area %	Rt
Acids	Oxiraneoctanoic acid, 3-octyl	18.51	45.80
Aldehyed	2,4-Heptadienal	2.46	5.50
Aromatic compound	4-[Phenylethynyl]acetophenone	47.78	28.81
Esters	6,7-Epoxypregn-4-ene-9,11,18-triol-3,20-dione,11,18-diacetate	2.11	12.40
	Dodecanoic acid,2,3-bis(acetyloxy) propyl ester	2.67	23.03
	trans-2-Phenyl-1,3-Dioxolane -4-Methyl-octa-dec-9,12,15-trienoate	2.68	27.56
	Oleic acid,3-(octadecyloxy)propyl ester	10.03	42.20
	Ethyl- iso-allocholate	2.55	52.21
	Testoster-3,11-dione,9-thiocyanato,acetate	2.60	60.31
Miscellaneous	t-Butyl{2[3(2,2-di-methyl-6-methylenecyclohexyl) propyl] [1,3]dithianyl}dimethylsilane	1.50	7.13
	Pyrrolidine,1-(1-cyclopenten-1-yl)	2.46	13.52
	5,11,17,23-Tetra-butyl-25,26,27,28-tetra-hydroxycalix-4-arene	1.51	14.41
	Dimethoxy-glycerol docosyl ether	1.56	59.74

Table 3: GC-MS analysis of fermented soy bean by *Tricoderma koningiF-27*

Chemichal Groups	Identified compounds	Area %	Rt
Acids	6-Fluoro-pyridine-2,3-di carboxylic acid	1.90	6.85
	Acetic acid, oxo	1.55	20.07
	9,12-Octadeca -dienoic acid (Z,Z)	4.23	46.10
Aromatic compound	4-[Phenylethynyl]acetophenone	55.20	28.81
Esters	Octa decanoic acid, 9,10-dihydroxy, methyl ester	3.23	6.51
	Carbonic acid, (ethyl)(1,2,4-tri-azol-methyl)di ester	2.48	6.68
	Cyclo propane-dodecanoic acid, 2-octyl, methyl ester	3.41	7.62
	5,8,11,14-Eicosa tetraenoic acid, ethyl ester	11.77	10.35
	7-Methyl-Z-tetradecen-1-ol-acetate	3.63	10.54
	Ethyl isoallocholate	2.57	52.21
Miscellaneous	Octa ethylene glycol monododecyl ether	2.26	5.67
	3-[2-Tetra hydropyranyloxy]-1-butyne	2.13	6.79
	Retinol, acetate	1.64	33.94
	Dimethoxy-glycerol-docosyl-ether	1.73	62.37

Table 4: GC-MS analysis of fermented soy bean by*Asperigillus fumigatusF-55*

Chemichal Groups	Identified compounds	Area %	Rt
Alcohols	Linalool	9.57	10.33
	24,25-Dihydroxy-cholecalciferol	4.97	13.50
Aromatic compound	3-phenyl -2,3-Epoxy cholestane	7.97	45.83
Cyclic hydrocarbon	2,3-Bis endo diacetyl-7-antiformyl-norbornene	56.80	30.68
Esters	Pentanoic acid,2,2dimethyl,1,2,3propanetriyl-ester	1.78	10.43
	[1,1'Bicyclopropyl]-2-octanoic acid,2'hexyl,methyl ester	3.06	27.56
	Ethyl iso allocholate	3.70	52.21
Miscellaneous	Zeaxanthin	1.94	35.33
	Lucenin 2	1.95	63.63
	Quercetin-7,3',4'-trimethoxy	1.54	28.35
	Hept-1-en-3-one	1.63	5.83

generated by fungal enzymatic actions on lipids and/or amino acids, or by the Maillard reaction [24, 25]. Although the flavour notes of ketones and esters are generally desirable, their aroma contributions might have been minimal since low levels of these compounds were quantified.

Erythritol Production: Current biotechnological production of erythritol use osmophilic yeasts like A*ureobasidium sp., Trichosporonoides sp., Torula sp.* and *Candida magnoliae*. As substrate a highly concentrated glucose (typically 40% (w/v)) solution is applied, which is gained from chemically and

Table 5: *In vitro* antioxidant activity (A.A) of ethanolic extract of row and fermented soy bean *by Trichoderma reeseiF.417*

	Inhibition %							
	A.A at different extract concentrations by β- carotene method				A.A at different extract concentrations by DPPH free radical scavenging method			
Products	50ug/ml	100ug/ml	200ug/ml	400ug/ml	50ug/ml	100ug/ml	200ug/ml	400ug/ml
Row soya bean extract	13.51±0.4	21.28±1.4	28.75±2.0	44.85±2.7	20.33±1.1	28.51±1.8	40.11±2.1	48.93±2.3
fermented soya bean extract	50.23±0.9	66.82±1.9	81.31±1.2	87.35±1.9	56.60±1.2	70.32±2.0	80.14±1.9	89.55±2.1
TBHQ*	75.2±3.1	85.0±2.5	94.0±2.7	99.5±2.7	76.53±2.3	83.75±2.5	95.36±2.6	99.73±2.6

*TBHQ: Tert-butyl hydroquinone, standard synthetic antioxidant

enzymatically hydrolyzed wheat- and cornstarch. The hydrolyzed starch serves as carbon source and causes a high osmotic pressure that pushes the yeast to produce the osmolyte erythritol [26]. It would be an interesting alternative to use organisms that can utilize waste soybean manufacturing products for the production of erythritol. In the present study we focused on the potential of producing erythritol in *T. reesei* F-417 from waste soybean manufacturing products compared to other microorganisms (*Penicillium funiculosum* F-229*, Tricoderma koningi* F-27*and Asperigillus fumigatus* F-55). Waldemar *et al*., [27] showed a possible microbial process for utilization of crude glycerol generated by the biodiesel industry for erythritol production. The results showed that *T.reesei*F.417 can produce glycerol (1,2,3-propanetriol) of soybeans, which in turnis used in the production of erythritol [27]. Crude glycerol is potentially suitable as a carbon source for large amounts of erythritol on a glycerol based substrate. The most obvious target of research dealing with the addition of value to glycerol by biotechnological means is in its biotransformation into 1,3- propanediol by several bacterial strains [28]. It is known that glycerol catabolism in respiratory-sufficient yeasts and fungus species occurs only via the glycolysis. The glycerol kinase pathway is responsible for glycerol degradation. The glycerol catabolic pathway includes phosphorylation by glycerol kinase and a subsequent oxidation by a flavin adenine dinucleotide (FAD)-dependent glycerol 3- phosphate dehydrogenase, located on the outer surface of the mitochondrial inner membrane. The dihydroxyacetone phosphate formed enters the glycolytic pathway [27]. In addition, the selected mutant of *Y. lipolytica* Wratislavia K1 produced large amounts of erythritol on a glycerol based substrate [27-29].Waldemar *et al*., [27] observed increase in the amount of erythritol showed that the starting glycerol concentration has a profound effect on the metabolism of glycerol by the *wratislavia K1* strain.

Our results showed GC-MS analysis of the cultivation broth of *T.reesei*F.417strains grown on waste soybean revealed especially accumulation of arabinitol and ribitol. Both substances are metabolites in the interconversion of the pentoses derived from lignocelluloses degradation (i.e. L-arabinose, D-xylose). On the other hand, the results proved that the fungus *Penicillium funiculosum* F-229 *Tricoderma koningi*F-27 and *Asperigillus fumigatus*F-55 can not produce erythritol. The results suggest that waste soybean manufacturing products can be regarded as an efficient substrate for the simultaneous biosynthesis of high amounts of erythritol by *T.reesei* F.417. Future research will try to use the best conditions to increase production erythritolusing *T.reesei F.417*.

Therapeutic Effect of Volatile Compounds: Our results showed (Table 5) that waste soybean manufacturing products by fermentation of *T.reesei* F.417 strains have high power as antioxidant compounds and therapeutic effect. Den Hartog *et al*. [30] reported that erythritol was shown to be an excellent $HO^{\cdot}$ radical scavenger and an inhibitor of 2,2'-azobis-2-amidinopropane dihydrochloride-induced hemolysis but inert toward superoxide radicals [30]. High-performance liquid chromatographic and electron spin resonance spectroscopy studies showed that the reaction of erythritol with hydroxyl radicals resulted in the formation of erythrose and erythrulose by abstraction of a carbon-bound hydrogen atom. Meanwhile, the polyunsaturated fatty acids n-Hexadecanoic acid, oleic acid and 9,12- octadecadienoic aconjugated linolic acid known as an antioxidant that can protect membranes from harm. These acids have also been reported in the reduction of coronary heart disease [9]. 9,12- octadecadienoic acid has the property of anti-inflammatory, hypocholesterolemic, cancer preventive hepatoprotective and antimicrobial as reported by Rajeswari *et al.*, [9]. Glycerol (1,2,3Propanetriol) is a potent

osmotic dehydrating agent with additional effects on brain metabolism. In doses of 0.25-2.0 g/kg glycerol decreases intracranial pressure in numerous disease states, including Reye's syndrome, stroke, encephalitis, meningitis, pseudotumor cerebri, central nervous system tumor and space occupying lesions. It is also effective in lowering intraocular pressure in glaucoma and shrinking the brain during neurosurgical procedures [31].

CONCLUSION

T. reesewstrains is very promising fungus for cheap production process of erythritol and antioxidant compounds from waste soybean manufacturing products.

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