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Short Communication

Microbial degradation of amygdalin of bitter apricot seeds (*Prunus armeniaca*)

M.J.R. Nout [a,*], G. Tunçel [b], L. Brimer [c]

[a] *Department of Food Science, Agricultural University, Bomenweg 2, 6703 HD Wageningen, The Netherlands*

[b] *Faculty of Engineering, Ege University, 35100 Bornova-Izmir, Turkey*

[c] *Department of Pharmacology and Pathobiology, The Royal Veterinary and Agricultural University, 13 Bülowsvej, DK-1870 Frederiksberg C, Copenhagen, Denmark*

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Abstract

Amygdalin is a cyanogenic glycoside occurring among others in almonds and bitter apricot seeds with interesting levels of dietary protein. Utilization of seeds for human or animal nutrition requires adequate detoxification. In the present paper, selected filamentous fungi (*Mucor circinelloides*, *Penicillium nalgiovense*) and yeasts (*Hanseniaspora valbyensis*, *Endomyces fibuliger*) were tested for their in-situ ability to decompose amygdalin. The latter (*Endomyces fibuliger*) was best able to grow on autoclaved bitter apricot seeds and detoxify them from 30 μMol CN/g dry matter to less than 1 μMol CN/g dry matter after 48 h of incubation at 27°C.

Keywords: Apricot seeds; Amygdalin; Cyanogenic; Detoxification; Fungi; Yeasts; *Endomyces fibuliger*

1. Introduction

Bitter apricot seeds (*Prunus armeniaca*) are by-products of the apricot processing industry. The seeds have a high protein content (20–25%, dry weight basis) and could be of interest as a food or feed ingredient. However, depending on variety, they contain approximately 50–150 μMol/g (dry weight basis) of the potentially toxic cyanogenic glycosides amygdalin and prunasin (Tunçel et al., 1990; Brimer et al., 1993). We reported earlier (Tunçel et al., 1990) on prospects for detoxification

* Corresponding author.

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by biotechnological means, i.e. by using the endogenous enzymes combined with microbial fermentation. The tempe fermentation process using the fungus *Rhizopus oligosporus* as inoculant enabled a removal of around 70% of total cyanide potential. As we considered this inadequate from toxicological point of view, we developed a simple method based on the picrate test (Guignard, 1906), as used by Brimer and Moelgaard (1986), to screen microbial strains for their ability to degrade amygdalin by hydrolysis (Brimer et al., 1993). The results obtained can be summarized as follows: of 29 strains of lactic acid bacteria, 16 were unable to degrade amygdalin while the maximum extent of degradation was 27% of the initial level after overnight incubation; of 21 yeast strains, six could not degrade amygdalin, and maximum degradation was 98%; of 25 strains of filamentous fungi, only 1 could not degrade amygdalin while several strains were found able to degrade up till 99% of the initial level of amygdalin (Brimer et al., 1993). This paper describes detoxification trials with those strains able to degrade $> 95\%$ of initial levels of amygdalin, i.e. strains of *Hanseniaspora valbyensis*, *Endomyces fibuliger*, *Mucor circinelloides* and *Penicillium nalgiovense*.

2. Materials and methods

2.1 Materials.

Bitter apricot seeds were obtained from Izmir, Turkey. *Hanseniaspora valbyensis* strain LU 263 (LU codes refer to the collection maintained at the laboratory of food microbiology, Agricultural University, Wageningen, the Netherlands), *Endomyces fibuliger* strain LU 677, *Mucor circinelloides* strain LU M40 and *Penicillium nalgiovense* strain LU 520 originate from fermented foods. All were grown and maintained on Malt Exctract Agar (Oxoid CM 59) slants. Incubation was at 28°C for 3–5 days, and grown cultures were stored at 5°C. *Bacillus* sp. strains B4 and B6 had been isolated from apricot seeds previously (Brimer et al., 1993). They were grown (30°C for 2–3 days) and maintained (5°C) on Plate Count Agar (Oxoid CM325) slants.

2.2. Reagents.

D-Amygdalin (Sigma St.Louis USA, A6005) was used for standard as a 1 mM solution in distilled water. Pectinase (EC 3.2.1.15) from *Rhizopus sp.* (Sigma St.Louis USA, P2401) was used as a source of hydrolytic enzymes (Brimer and Rosling, 1993) for degradation of amygdalin standards, as a 0.2% w/v solution in distilled water in the quantification of total cyanide potential. Picrate reagent sheets were prepared according to Brimer et al. (1983).

2.3. Methods.

Extraction of amygdalin and degradation products: 10 g sample (seed dry weight) was homogenized with 90 ml 0.1 M ortho-phosphoric acid (Cooke, 1978) in

a glass blender jar (Braun Multimix MX32 type 4207 Germany) as follows: 15 s speed 1, 1 min speed 3, 1 min rest, 1 min speed 3. Homogenates (A) were found stable for 7 days at 4°C as also found for cassava extracts by Cooke (1978).

Amygdalin degradation. To 5 ml homogenate (A), 15 ml phosphate buffer (0.1 M Na_3PO_4/H_3PO_4, pH 7) is added to give homogenate (B) with pH 6.5. In a microtiter plate, the following wells are prepared: (a) amygdalin standard (6 wells/plate): 10, 20 or 30 μL amygdalin + 100 μL pectinase + distilled water totalling 200 μL; (b) concentrated sample for degradation products (HCN and cyanohydrins) (2 wells/plate): 100 μL sample homogenate (B) + 100 μL dist. water; (c) diluted sample for degradation products (2 wells/plate): 10 μL sample homogenate (B) + 190 μL dist. water; (d) concentrated sample for total cyanide potential (intact amygdalin + degradation products) (2 wells/plate): 100 μL sample homogenate (B) + 100 μL pectinase; (e) diluted sample for total cyanide potential (2 wells/plate): 10 μL sample homogenate (B) + 90 μL dist. water + 100 μL pectinase. A picrate reagent sheet (plastic side up) is placed on top of the microtiter plate and weighed down gently with a flat object of approx. 100 g, and incubated at 25°C overnight. In this way, one microtiter plate accommodates a 3-level amygdalin calibration and three different samples.

Assessment. The density of the red-brown spots on yellow background typical of positive reactions, was assessed by measuring the absorption of transmitted light at 550 nm using a microtiter plate reader (EAR 400, SLT-Labinstruments, A-5082 Groedig, Austria) (Brimer et al., 1993). Using the amygdalin standards for calibration in each picrate sheet, extinctions of either dilute or concentrated sample which were closest to the standard, were used for calculations. Degradation products labeled as 'cyanohydrins' were based upon wells type (b) or (c), and the difference of total cyanide potential (wells type 'd' or 'e') and degradation products was labeled as 'amygdalin'. Both were expressed as μmol CN/g sample dry weight. The detection level was 1 μmol HCN/g dry weight.

Seed processing. Apricot seeds were rinsed 3 times with tapwater, and ground coarsely in a Fritsch mill (Fritsch Pulverisette type 14.702, Germany) (fine rotor, no sieve, speed 1). Particle size distribution of the grits was: 24% < 1.0 mm; 20% 1.0–2.0 mm; 36% 2.0–4.8 mm; 20% > 4.8 mm. The ground seeds were soaked overnight at 22°C in tapwater at a rate of seeds: liquid = 1:3 w/v. Subsequently, the material was recovered on a sieve and washed twice with tapwater, and either (a) cooked in fresh tapwater (seeds:water = 1:3 w/v) at 100°C for 30 min with open pan or (b) dispensed without further addition of water in screw-capped glass jars and autoclaved at 121°C for 30 min. After cooling, the autoclaved material was inoculated with the microbial strains tested at a rate of approximately 10^5–10^6 cfu/g, and incubated with loose-fitting screw-caps in order to avoid anaerobiosis.

Microbial enumeration. The growth of bacteria was monitored using pour-plates with Plate Count Agar (Oxoid CM325) incubated at 30°C for 3 days; yeasts were

counted after 3–5 d at 25°C in pour-plates of Yeast Extract Glucose Agar (Oxoid CM545) to which 0.01% (w/v) sterile oxytetracycline (Oxoid SR73) was added after autoclaving; and filamentous fungi were enumerated using surface-inoculated plates with Rose Bengal Agar modified according to Baggerman (1981), incubated at 28°C for 5 days.

3. Results and discussion

Table 1 shows the effect of preparatory treatments (grinding, soaking, heating) on cyanide compounds in bitter apricot seeds. Although the initial cyanide content of the present batch of seeds is much higher, these data show a similar trend as observed earlier (Tunçel et al., 1990), indicating that endogenous β-glycosidase activity causes significant degradation of amygdalin in soaked ground seeds. The resulting cyanohydrins volatize during cooking, whereas the remaining amygdalin is practically unaffected. Autoclaved soaked seeds still contained detectable cyanohydrins, probably due to restricted volatization in the closed autoclave vessel. When non-inoculated cooked (100°C, 30 min) seeds were kept in jars with loose-fitted screw-caps at 4°C for 48 h, no microbial growth occurred and the cyanide content did not change. However, when kept under similar conditions but incubated at 30°C for 48 h, profuse bacterial growth occurred ($Log_{10}N/g = 9.6$ on plate count agar) and cyanide was removed till below detection level (1 μmol/g dm) (data not shown). In similar investigations carried out previously, six out of 10 tested purified isolates all belonging to *Bacillus* sp. were able to degrade amygdalin in aqueous solution (Brimer et al., 1993). In these investigations, strains B4 and B6 resembling *B. subtilis* degraded 85% and 100%, respectively, of amygdalin overnight. These strains were included in the detoxification trials. When non-inoculated autoclaved (121°C, 30 min) seeds (Table 1) were incubated at 27°C for 72 h, no microbial growth occurred and the cyanide content remained constant. The latter treatment was therefore chosen to prepare seeds for detoxification trials with microbial pure cultures.

In Table 2, the effect of selected microbial cultures on cyanide levels in prepared bitter apricot seeds is shown. Not all cultures which can degrade

Table 1
Effect of pre-fermentation treatments on cyanide content of bitter apricot seeds.

	Cyanohydrins [a]	Amygdalin [a]
Raw seeds	6 (0.5) [b]	118 (29)
Ground, soaked overnight	24 (4)	30 (2)
Soaked + Cooked in open pan (100°C, 30 min)	ND	28 (2)
Soaked + Autoclaved (121°C, 30 min)	9 (1)	32 (3)

[a] μmol HCN equivalents/g dry weight; [b] Duplicates from two independent batches of seeds (mean and standard deviations; n = 4); ND, not detected (< 1 μmol HCN equivalents/g dry weight).

Table 2
Effect of aerobic incubation at 27°C with selected microorganisms on cyanide content of soaked and autoclaved bitter apricot seeds.

Strain Code		Incubation period (h)			
		0	24	48	72
Bacillus sp.					
LU B4	CFU [a]	6.86	7.49	8.82	9.65
	Cyanohydrins [b]	9	5	2	9
	Amygdalin [b]	32	39	13	10
Bacillus sp.					
LU B6	CFU	6.81	7.48	9.05	9.90
	Cyanohydrins	9	4	2	11
	Amygdalin	32	30	11	7
Hanseniaspora valbyensis					
LU 263	CFU	5.45	5.40	6.21	6.36
	Cyanohydrins	9	8	6	10
	Amygdalin	31	30	30	28
Mucor circinelloides					
LU M40	CFU	5.10	4.84	4.67	4.42
	Cyanohydrins	10	6	9	7
	Amygdalin	32	32	28	27
Penicillium nalgiovense					
LU 520	CFU	4.94	4.20	4.12	4.40
	Cyanohydrins	9	10	8	4
	Amygdalin	30	31	29	26
Endomyces fibuliger					
LU 677	CFU	5.52	5.54	7.64	6.90
	Cyanohydrins	9	21	13	9
	Amygdalin	32	20	2	ND [c]
None (control)					
	CFU	< 1.7			< 1.7
	Cyanohydrins	9			1
	Amygdalin	30			31

[a] Colony Forming Units expressed as $Log_{10}N/g$ fresh sample; [b] μmol HCN equivalents/g dry weight; [c] ND, not detected (< 1 μmol HCN equivalents/g dry weight).

amygdalin in aqueous solution in a screening test (Brimer et al., 1993) can do so in-situ. The unsatisfactory results with *H.valbyensis*, *M.circinelloides* and *P.nalgiovense* are due to their poor growth on the substrate. Agar diffusion and auxanogram tests (data not shown) revealed that this was neither caused by water-soluble inhibitory compounds in the seeds, nor by C or N deficiency. Other possible reasons (e.g. poorly soluble phenolic compounds with antimicrobial effect) were not investigated. On the other hand, *Bacillus* sp. B4 and B6 grew well and caused significant removal of cyanide. However, detectable residues remained. The best results were obtained with *Endomyces fibuliger* LU 677 which showed good growth and resulted in complete removal of cyanide after 3 days of incubation. This yeast was isolated from Indonesian 'Ragi', a traditional inoculum for the fermentation of glutinous rice into the product 'Tape Ketan'. *E.fibuliger* is

considered as functional flora during this fermentation and contributes to the aroma of the fermented rice (Hesseltine et al., 1988). Further investigation of the effect of *E.fibuliger* on the chemical and organoleptic properties of bitter apricot seeds will be undertaken.

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