UDC 579.64:57.088

doi: 10.15389/agrobiology.2016.2.204rus
doi: 10.15389/agrobiology.2016.2.204eng

ALFP-FINGERPRINTING FOR CERTIFICATION OF *Aspergillus niger* L-4, THE CITRIC ACID COMMERCIAL PRODUCER

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Received January 11, 2016

Abstract

Citric acid plays an important role in cellular respiration, and participates in oxidation processes as a natural antioxidant and synergist antioxidant, inhibiting plant oxidoreductase, together with ascorbic acid. Citric acid is effective against east and bacterial pathogens, and can be used in ensilaging. Citric acid is an antimicrobial agent alternative to fodder antibiotics prohibited in European countries. It is metabolized in plants, animals and humans with no adverse effect. *Aspergillus niger* is the citric acid common producer. So far as cultural and morphological features are not enough to confirm strain authenticity essential for effective commercial product manufacturing, we used genetic certification of the strain. Note, in 2015 in wide range genetic study of *Aspergillus flavus* diversity the specific ALFP patterns were found though their practical aspects were not discussed (D. Singh et al., 2015). Herein, we studies osmophilic commercial strain *Aspergillus niger* L-4, the citric acid producer derived due to chemical and UV mutagenesis and spontaneous mutations, using optimized AFLP-fingerprinting with 12 different primers. Specific AFLP patterns were obtained for *Aspergillus niger* L-4 authentication. Mse_cc GATGAGTCCTGAGTAACC and Eco_ac (FAM) GACTGCGTACCAATTAC primers were shown to be optimal providing maximum number of DNA fragments in the range of 33.68 to 593.78 bp (89 fragments) subject to the described method of fingerprinting and computer processing. The primers are effective regardless of sample volume. The resulting profiles can be used to authenticate the strain *Aspergillus niger* L-4 from different sources.

Keywords: *Aspergillus niger*, citric acid producer, genetic profiling, AFLP-fingerprinting

Citric acid plays the important role in biochemical reactions of cellular respiration, the biological significance of which is most weighty compared to other biochemical processes in cells. It is involved in oxidation as natural antioxidant and a synergist of antioxidants, inhibiting, along with ascorbic acid, oxidoreductases in plant tissue [1, 2]. Citric acid binds Fe^{+3} ions in colorless potato tubers improving taste. In the course of long-term storage of tuber crops at low temperature ($-4\text{ }^{\circ}\text{C}$ and $-8\text{ }^{\circ}\text{C}$) malic, fumaric and tartaric acids levels decrease where as citric acids increases [3]. At positive temperatures the antioxidative activity in plant tissues is not enough to slow down oxidation, so this necessitates using preparations with antioxidant activity. Citric acids is effective in control of contamination with east and bacterial pathogens in food products [4, 5]. Due to acidic conditions citric acid provides binding bivalent cations of bacterial oxidoreductases to chelates [5], it can be easy applied and removed from seeds, and is used to treat seeds for neutralization of fungal and bacterial pathogens for safe long-term storage [6]. So citric acid is considered effectively alternative to antimicrobial preparations with other acting mechanism, particularly, feed antibiotics prohibited in Europe. Products of citric acid metabolism in the body have no adverse effect.

Since citric acid synthesis occurs in each living things, it seems biotechnologically reasonable to consider tricarboxylic acid cycle (or Krebs cycle, TCC)

as universal source of citric acid. In microorganisms biochemical reactions are highly labile. Naturally microbes do not produce an excess of metabolites, though it may be due to changed parameters of incubation, or a proportion of gene induction and repression. At that, a resulting shift in the character of biochemical pathways, including TCC, occurs.

Micromycete *Aspergillus niger* is a common producer of citric acid, and its authenticity must be under control to provide stable production parameters. For commercial use the high producing strains of *A. niger* are derived which meets normative technological indicators [7]. However, these are not enough to ascertain the strain authenticity. A set of cultural and morphological traits is not sufficient to provide a complete assessment of individual strain within a biological species. Genetic uniformity is characteristic of commercial aspergillus strains producing citric acid, so that necessitate producing periodically pure cultures (elite lines) from a typical conidia or colony [7]. Thus, a genetic authenticity becomes of special importance. AFLP (amplified fragments length polymorphism) fingerprinting is a common technique in molecular genetics of microorganism. Using AFLP-fingerprinting, the differences have been successfully found between close related strains in different taxonomic groups including rhizobia [8-12], lactobacteria [13-16], and myceliar fungi *Trichoderma* and *Aspergillus* [17-20]. AFLP-fingerprinting is high reproducible, so it may be considered promising to authenticate microorganisms [21]. Each strain produces a unique pattern of amplified DNA fragment (AFLP-pattern), which is suitable for computerized processing to compare with other strain and can be used as «genetic passport». Earlier we have optimized the AFLP-fingerprinting protocol for strains of agricultural microorganisms [22].

Cultural and morphological features of *A. niger* strains have been shown not enough to identify individual specific traits [7]. RAPD-PCR (random amplification of polymorphic DNA) with universal primers AS15inv, L 15/AS19 and AA2c producing specific sets of genome DNA amplicons, was more effective for genotyping selected strains in this species [7].

Herein, the AFLP-patterns for osmophilic commercial strain *Aspergillus niger* L-4, the commercial producer of citric acids, are first reported. Specific primers, Mse_cc GATGAGTCCTGAGTAACC and Eco_ac (FAM) GACTGC-GTACCAATTAC, are optimized to produce 89 fragments ranged from 33.68 to 593.78 bp in size.

Our aim was a genome AFLP-fingerprinting of commercial citric acid producer *Aspergillus niger* L-4 using 12 combination of primers to obtain unique «genetic passport» for strain authentication.

Technique. Conidia of osmophilic commercial strain *Aspergillus niger* L-4 (collection of All-Russian Research Institute for Food Additives) were the biological material.

For DNA isolation conidia were thoroughly homogenized in mortar with pestle, then frozen, thawed and homogenized again (the procedure was repeated 4 times). After an aliquot 500 µl 2× CTAB buffer (2 % CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0) was added, it was placed in 1.5 ml eppendorf and incubated 60 min at 65 °C using vortex for periodical shaking. For extraction repeated twice the chloroform:isoamyl alcohol (24:1, v/v) was used. DNA was precipitated with an equal volume of isopropanol (5 min at room temperature), centrifuged (2 min, 14000 min⁻¹), the supernatant was air dried. Then the precipitate was dissolved in 200 µl 70 % ethanol, centrifuged 7 s, and alcohol was removed. Air dried DNA precipitate was suspended in 50 µl H₂O(MQ). Isolated DNA was stored at -20 °C.

DNA purification was performed by 1 % agarose gel electrophoresis in

a Sub Cell GT (Bio-Rad, USA) chamber for 60 min at 100 V. λ /HindIII Marker (0.5 $\mu\text{g}/\mu\text{l}$, Fermentas, USA; 1 μl) and DNA Gel Loading Dye (Thermo Scientific, USA; 5 μl) was added to the probes. DNA-containing slice was cut out of the gel and placed into eppendorf. Three volumes (to gel slice volume) of agarose solvent (3 M GITS, 20 mM EDTA, 10 mM Tris-HCl, pH 6.8; Triton X-100 to 40 mg/ml) was added to final volume of 500 μl . Tubes were incubated in thermostat at 65 °C for 5 min, stirring occasionally. To the dissolved gel slice the Silica reagent (Fermentas, USA; 40 μl) was added, and the probe was vortexed for 15 min and then centrifuged for 1 min at 2700 min^{-1} . After the first centrifugation the supernatant was poured out, after second it was removed using needle, and finally it was completely dried. Dry precipitate was rinsed in a 250 μl solution containing 25 % ethanol, 25 % isopropanol, 100 mM NaCl, pH 8.8) and centrifuged 1 min at 2700 min^{-1} . Liquid was poured out dry, and the precipitate was rinsed in 200 μl 96 % ethanol with the supernatant removing. The residue was dried for 10-15 min to evaporation of the alcohol and dissolved in 15 μl eluting solution (10 mM Tris-HCl, pH 8.0). After 5 min incubation at 65 °C and 15 min vortexing at room temperature, followed by one more incubation for 5 min at 65 °C, the samples was centrifuged 1 min at 14000 min^{-1} with removing supernatant to eppendorfs. To assay DNA concentration the 1 % agarose gel electrophoresis at 100 V for 60 min was used. A 3 μl DNA sample with 1 μl loading dye were poured per well; λ /HindIII Marker (0.5 $\mu\text{g}/\mu\text{l}$) was a marker (1 μl marker and 5 μl dye were mixed).

Aliquot 10 μl of purified DAN was subjected to restriction and ligation using 2.5 U EcoRI and MseI (Thermo Scientific, USA); 2.5 U T₄ ligase (Thermo Scientific, USA); two oligonucleotide adapters for each site adEco1 CTCGTAGAC-TGCGTACC and adEco2 AATTGGTACGCAGTCTAC for EcoRI, adMse1 GACGAGAGTCCTGAG and adMse2 TACTCAGGACTCAT for MseI (5 pmol each); the mixture was allowed for 18 hours at 37 °C. T₄ DNA ligase reaction buffer (Thermo Scientific, USA) was used.

For final fingerprinting, to aliquot 4 μl reaction mixture at the stage of restriction and ligation the 2.5 μl 10 $\times$ polymerase buffer, 2.5 μl 1,5 mM dNTPs, two selective primers (10 pmol each), of which one was FAM-marked, 1 U Taq DNA polymerase (Fermenta», USA) were added. All possible primer combinations were tested — Mse_0 GATGAGTCCTGAGTAA, Mse_c GATGAGTCCTGAGTAAC, Mse_g GATGAGTCCTGAGTAAG, Mse_t GATGAGTCCTGAGTAAT, Mse_ct GATGAGTCCTGAGTAACT, Mse_cc GATGAGTCCTGAGTAACC for MseI; Eco_0 (FAM) GACTGCGTACCAATT, Eco_ac (FAM) GACTGCGTACCAATTAC for EcoRI. Amplification was carried out in a T100 Thermal Cycler (Bio-Rad, USA) according to the protocol: 50 °C 5 s, 60 °C 5 s, 70 °C 2 min, 95 °C 1 min 30 s; 94 °C 30 s, 55 °C 30 s, 72 °C 1 min 30 s (34 cycles); 72 °C 2 min.

Preliminarily, a 3 μl DNA sample with 1 μl loading dye were analyzed by 3 % agarose gel electrophoresis (100 V for 3 hours, GeneRuler 100bp DNA Ladder, 0.5 $\mu\text{g}/\mu\text{l}$, Fermentas, USA, as molecular weight marker).

Capillary electrophoresis was performed using a genetic analyzer ABI3500xl (Applied Biosystems, USA) with an integral molecular weight marker GeneScan-600 LIZ Size Standard (Applied Biosystems, USA). DNA aliquots 0.1, 0.5, 1.0 and 2.0 μl were analyzed. After electrophoretic separation of fragments data were processed using BIONUMERICS 7.5 program (Applied Maths, USA).

Results. Strain L-4 was isolated after combined treatment of *A. niger* L-1 (collection of All-Russian Research Institute for Food Additives) with 1,4-bis-diazoacetil butane (13 % solution, 3 hours) and UV-irradiation at 3.3000 erg

per mm [23]. The strain produces citric acid at no less than $10 \text{ g} \cdot \text{dm}^{-3} \cdot \text{day}^{-1}$ on molasses-containing medium, highly acidize sucrose-mineral medium under fermentation (no less than $16 \text{ g} \cdot \text{dm}^{-3} \cdot \text{day}^{-1}$) and media containing corn, potato, wheat, rye or sorghum starch hydrolisate (no less than $18 \text{ g} \cdot \text{dm}^{-3} \cdot \text{day}^{-1}$). The strain is permanently (ones a year) re-cultured on must-agar, and additionally stored as dry conidia preparation (a 10 % residual moisture) for 9 months at 16–20 °C, and as dry conidia (a 10 % residual moisture) with sporulating mycelium at –18 to –20 °C with re-culturing each 2 years. Long-term storage at –80 °C is provided using Station of low temperature automatic storage of biological samples (Liconic Instruments, Liechtenstein) at RCAM collection (All-Russian Research Institute for Agricultural Microbiology) [24].

Based on preliminary estimation of the obtained patterns, the Mse_ct GATGAGTCCTGAGTAACT and Eco_ac (FAM) GACTGCGTACCAATTAC; Mse_cc GATGAGTCCTGAGTAACC and Eco_ac (FAM) GACTGCGTACCAATTAC; Mse_g GATGAGTC-CTGAGTAAG and Eco_ac (FAM) GACTGCGTACCAATTAC were the most effective primers of those 12 tested as producing the highest number of DNA fragments.

The results of the automatic capillary electrophoresis (Fig.) and results of analysis formalized on amplified fragments' size for each variant (summarized in the table) are shown hereinbelow.

AFLP patterns of *Aspergillus niger* L-4 DNA obtained with the best three pairs of primers (automatic capillary electrophoresis): AC_CC; AC_CT and AC_G — primers Mse_cc/Eco_ac, Mse_ct/Eco_ac and Mse_g/Eco_ac, respectively; 0.1, 0.5, 1.0 and 2.0 — volume of tested DAN sample (in μl). An analyzer ABI3500xl, Applied Biosystems, USA) with an integral molecular weight marker GeneScan-600 LIZ Size Standard (Applied Biosystems, USA).

Results of *Aspergillus niger* L-4 AFLP-fingerptning formalized on amplified fragments' size for the best three pairs of primers

Fragment, bp	Mse cc/Eco ac		Mse ct/Eco ac		Mse g/Eco ac	
	AC CC 0.1	AC CC 2.0	AC CT 0.1	AC CT 2.0	AC G 0.1	AC G 2.0
593.78	1	1	1	1	1	1
590.69	1	1	1	1	1	1
588.22	1	1	1	1	1	1
580.43	1	1	1	1	1	1
578.20	1	1	0	1	1	1
573.78	1	1	1	1	1	1
571.43	1	1	1	1	1	1
569.00	1	1	1	1	1	1
565.78	1	1	1	1	1	1
562.58	1	1	0	0	1	1
556.26	1	1	0	0	1	1
549.43	1	1	0	0	1	1
545.95	1	1	0	0	1	1
543.39	1	1	0	0	1	1
540.70	1	1	0	0	1	1
534.38	1	1	1	1	1	1
528.60	1	1	1	1	1	1
522.89	1	1	1	1	1	1
518.91	1	1	0	0	1	1
517.94	1	1	1	1	1	1
516.98	1	1	1	1	1	1
515.58	1	1	1	1	1	1
509.15	1	1	1	1	1	1
497.64	1	1	1	1	1	1
493.11	1	1	1	1	1	1
489.14	1	1	1	1	1	1
486.67	1	1	0	0	1	1
483.85	1	1	1	1	1	1
482.60	1	1	1	1	1	1
478.11	1	1	1	1	1	1
471.57	1	1	0	1	1	1
468.29	1	1	0	1	1	1
465.09	1	1	0	0	1	1
461.17	1	1	0	0	1	1
457.84	1	1	1	1	1	1
453.14	1	1	0	0	1	1
449.77	1	1	0	0	1	1
443.78	1	1	1	1	1	1
439.16	1	1	0	0	1	1
435.39	1	1	1	1	1	1
429.90	1	1	0	0	1	1
424.21	1	1	1	1	1	1
418.49	1	1	1	1	1	1
417.20	1	1	0	0	1	1
415.40	1	1	0	0	1	1
409.27	1	1	0	0	1	1
399.10	1	1	0	0	1	1
392.88	1	1	0	0	1	1
388.44	1	1	0	1	1	1
384.48	1	1	1	1	1	1
375.76	1	1	0	0	1	1
356.98	1	1	0	0	1	1
346.87	1	1	0	0	1	1
339.88	1	1	0	0	1	1
334.86	1	1	0	0	1	1
326.17	1	1	0	0	1	1
320.14	1	1	1	1	1	1
318.12	1	1	0	0	1	1
316.96	1	1	0	0	1	1
316.00	1	1	0	0	1	1
313.58	1	1	0	0	1	1
310.47	1	1	0	0	1	1
298.28	1	1	0	0	1	1
285.23	1	1	0	0	1	1
273.72	1	1	0	0	1	1
268.38	1	1	1	1	1	1
263.05	1	1	0	0	1	1
256.30	1	1	0	1	1	1
252.72	1	1	1	1	1	1

	Table continued					
250.88	1	1	0	0	1	1
247.40	1	1	0	0	1	1
244.32	1	1	0	0	1	1
238.09	1	1	1	1	1	1
230.24	1	1	0	1	1	1
219.86	1	1	1	1	1	1
217.75	1	1	0	0	1	1
216.46	1	1	1	1	1	1
212.77	1	1	1	1	1	1
192.39	1	1	0	0	1	1
183.90	1	1	0	0	1	1
172.12	1	1	1	1	1	1
152.41	1	1	0	1	1	1
141.73	1	1	1	1	1	1
130.64	1	1	1	1	1	1
117.54	1	1	1	1	1	1
116.81	1	1	1	1	1	1
53.37	1	1	1	1	0	0
34.85	1	1	0	0	0	0
33.68	1	1	0	0	0	0

Note. AC_CC, AC_CT and AC_G — primer pair Mse_cc/Eco_ac, Mse_ct/Eco_ac and Mse_g/Eco_ac, respectively; 0.1 and 2.0 — sample volume, µl.

Thus, the optimal pair of primers was Mse_cc GATGAGTCCTGAG-TAACC and Eco_ac (FAM) GACTGCGTACCAATTAC, which produce the highest fragment number — 89 fragments, from 33.68 to 593.78 bp in size, provided the suggested technique and processing results. For each pair of primers the AFLP patterns do not depend on the volume of the sample. Note, in 2015 in large scale investigation of biodiversity of *Aspergillus flavus* strain the unique AFLP patterns have been found, however, the practical aspects of this phenomenon was not under consideration [25].

Thus, for the first time the AFLP-fingerprinting was optimized and used for molecular authentication of micromycete *Aspergillus niger* L-4 commercial strain. Obtained AFLP patterns can be used for authentication of this strain from different sources provided the place and protocol of assessment are the same as described herein. Developed method may be applied to produce «genetic passports» for other *Aspergillus* strains.

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