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SHORT COMMUNICATION

Novel *Halomonas* sp. B15 isolated from Larnaca Salt Lake in Cyprus that generates vanillin and vanillic acid from ferulic acid

Ioannis Vyrides[1] · Maria Agathangelou[1] · Rodothea Dimitriou[1] · Konstantinos Souroullas[1] · Anastasia Salamex[1] · Aristostodimos Ioannou[1] · Michalis Koutinas[1]

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Abstract Vanillin is a high value added product with many applications in the food, fragrance and pharmaceutical industries. A natural and low-cost method to produce vanillin is by microbial bioconversions through ferulic acid. Until now, limited microorganisms have been found capable of bioconverting ferulic acid to vanillin at high yield. This study aimed to screen halotolerant strains of bacteria from Larnaca Salt Lake which generate vanillin and vanillic acid from ferulic acid. From a total of 50 halotolenant/halophilic strains 8 grew in 1 g/L ferulic acid and only 1 *Halomonas* sp. B15 and 3 *Halomonas elognata* strains were capable of bioconverting ferulic acid to vanillic acid at 100 g NaCl/L. The highest vanillic acid (365 mg/L) at these conditions generated by *Halomonas* sp. B15 which corresponds to ferulic acid bioconversion yield of 36.5 %. Using the resting cell technique with an initial ferulic acid concentration of 0.5 g/L at low salinity, the highest production of vanillin (245 mg/L) took place after 48 h, corresponding to a bioconversion yield of 49 %. This is the first reported *Halomonas* sp. with high yield of vanillin production from ferulic acid at low salinity.

Keywords Bioconversion · Ferulic acid · Larnaca Salt Lake · Resting cell · Vanillin · Vanillic acid

✉ Ioannis Vyrides
Ioannis.vyrides@cut.ac.cy

[1] Department of Environmental Science and Technology, Cyprus University of Technology, 30 Archbishop Kyprianos, 3036 Lemesos, Cyprus

Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) and associated metabolites are of considerable economic importance because of increasing popularity and price. However, vanillin is often produced in environmentally unfriendly processes and lacks substrate selectivity. Moreover, according to the European legislation (Directive 88/388/EEC), the use of this chemically produced vanillin is restricted in food, beverages and cosmetics. Natural vanillin is desirable in the worldwide market despite its higher prices. Nevertheless, natural vanillin production by direct extraction from vanilla is subject to many problems, such as low concentration, making the extraction process expensive (Serra et al. 2005; Zamzuri et al. 2014).

A promising and emerging field for the production of natural flavours is through microbial production. Therefore, the use of microorganisms that can biotransform "cheap substances" such as ferulic acid (FA) to high value added products, such as vanillin, is an alternative economical route (Xu et al. 2007). FA is one of the most abundant phenolic compounds; it occurs in lignocellulose and in plant cellular walls as well as in the cell walls of agricultural crops such as wheat, maize and sugar (Ashengroph et al. 2012).

Several studies found that the vanillin produced from microorganisms is either rapidly converted to other products or used by the microorganism as a source of carbon and energy. Overhage et al. (1999) and Calisti et al. (2008) found that ferulic acid is first activated to feruloyl-CoA by a feruloyl-CoA synthetase and then the CoA thioester is subsequently hydrated and cleaved to vanillin and acetyl-CoA by an enoyl-CoA hydratase/aldolase. Abdelkafi et al. (2006, 2008) using *Halomonas elognata* strains found that the ferulic acid was rapidly transformed to vanillic acid at a

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high yield (80 %). In these studies (Abdelkafi et al. 2006, 2008) however, no vanillin was produced which has a considerably higher value added product than vanillic acid. Moreover, until now limited microorganisms have been found capable of bioconverting ferulic acid to vanillin at high yield (Table 1). As shown in Table 1, so far only *Streptomyces* sp., *Amycolatopsis* and recombinants *Pseudomonas fluorescens* BF13 as well as *P. fluorescens* BF13 produced substantial amounts of vanillin from ferulic acid (Table 1). Furthermore, according to a recent review by Yin et al. (2014) halophilic microorganisms present a significant potential for biotechnological applications and therefore these microorganisms have been investigated in Larnaca Salt Lake for their potential to produce vanillin.

This study aimed to isolate halotolerant bacteria from Larnaca Salt Lake in Cyprus that can biotransform ferulic acid to vanillin at high yield.

Materials and methods

Larnaca Salt Lake Complex is one of the most important natural standing water bodies in Cyprus and is of international ecological significance, declared as a protected area by a decision of Council of Ministers (1997), Ramsar Site, Natura 2000 Site. The salt water penetrates the porous rock between the lake and the sea, making the water very salty (20–300 g NaCl/L) (Department of Fisheries and Marine Research Cyprus). Despite some studies on the ecology of the Larnaca Salt Lake complex, until today no study has investigated the microbial ecology of the lake.

In order to have homogenous and representative samples, 16 samples were collected from Larnaca Salt Lake. The samples were collected at 2–5 cm depth from the surface during July and August 2012. They were used as an

Table 1 Studies examined the bioconversion of ferulic acid to vanillin and vanillic acid

Microorganisms	System, conditions	Initial ferulic acid	Maximum vanillin (yield)	Maximum vanillic acid (yield)	References
Recombinant *P. fluorescens BF13*	3L stirred tank reactor 3 g/L wet weight	10 mM	8.01 mM in 24 h. Yield: 81 %	1.6 mM in 24 h. Yield: 16 %	Di Gioia et al. (2010)
Recombinant *Pseudomonas putida strain KT2440*	Resting cell (5 ml) 200 rpm, 30 °C	10 mM	8.6 mM in 3 h. Yield: 86 %	–	Graf and Altenbuchner (2014)
First ferulic acid addition to *Aspergillus niger K8* and its supernatant to *Phanerochaete crysosporium ATCC 24725*	Fed batch (150 rpm, 30 °C and 120 ml in 250-ml flask)	0.3 g/L	44.8 mg/L Vanillin in 60 h. Yield: 14.9 %	116.9 mg/L in 36 h. Yield: 36.9 %	Motedayen et al. (2013)
Enterobacter sp. *Px6-4*	Fed batch	1 g/L	17.6 mg/L in 108 h. Yield: 1.76 %	–	Li et al. (2008)
Amycolatopsis HR167	Fed batch	5.1 mM	2.6 mM after 6.5 h. Yield: 50 %	1.5 mM in 4.5 h. Yield: 29 %	Achterholt et al. (2000)
Streptomyces sp. *strain V-1*	Fed batch (200 rpm, 30 °C and 30 ml in 300-ml flask)	9 g/L	5.24 g/L in 18 h. Yield: 58 %	–	Hua et al. (2007)
Streptomyces setonii	Fed batch (culture pre grown on	8 g/L	3.8 g/L in 26 h. Yield: 47 %	0.2 g/L in 26 h	Muheim and Lerch (1999)
Streptomyces halstedii	Resting cells	1 g/L	0.08 g/L in 8 h. Yield: 8 %	0.8 g/L in 24 h. Yield 80 %	Brunati et al. (2004)
			8 %		
Bacillus licheniformis SHL1	Resting cells, 25 ml in 125-ml flask at 37 °C, 200 rpm	1 g/L	52 mg/at 30 h. Yield: 0.05 %	0.495 g/L in 45 h. Yield: 49.5 %	Ashengroph et al. (2012)
Halomonas Elongatastrain Mar	Resting cell 4 g/L wet biomass, Salinity: 80 g NaCl/L	5 mM	–	4.3 mM in 14 h. Yield: 86 %	Abdelkafi et al. (2006)
Halomonas elongata DSM 2581	Resting cell 5 g/L wet biomass, Salinity: 80 g NaCl/L	10 mM	–	3.95 mM in 10 h. Yield: 79 %	Abdelkafi et al. (2008)

inoculum for the enrichment culture, which was cultivated in a sterilized salt medium (SM) with composition: 2 g/L $KHPO_4$, 5 g/L $MgSO_4 \cdot 7H_2O$, 0.2 g/L NH_4Cl, 4 g/L yeast extract, 4 g/L peptone, 1 g/L saccharose and 200 g/L NaCL Enrichment of microorganisms was carried out in 250 mL flasks and collected samples (about 5 g) were added at a total volume of 100 mL (SM). The samples were inoculated for 3 weeks at 30 °C at pH 7. Isolation and characterization of halotolenat/halophilies degrading bacteria was performed by the streak method on enrichment medium containing the same media SM as enrichment and agar. The inoculated petri dishes were incubated for 14 days at 30 °C. After incubation, 50 single colonies were picked and re-cultivated in the same agar medium following the same procedure as stated above. The ability of individual colonies (50) to grow at 1 g/L ferulic acid at 100 g NaCl/L was tested in petri dishes. Out of 50 strains, 8 strains grew in 1 g/L ferulic acid after 1 week.

Then, the DNA was extracted from the 8 single colonies according to Drakou et al. (2015). After extraction, a PCR reaction took place using the following primers: (1) 8f: 5′ AGA GTT TGA TCC TGG CTC AG 3′ and (2) 1542R: 5′-AAG GAG GTG ATC CAG CCG CA 3′. The reaction was carried out according to the following protocol: 94 °C (2 min) followed by 33 cycles consisting of 94 °C (1 min), 56 °C (1 min), 72 °C (2 min) and 72 °C (7 min). Sequencing alignment was performed by Macrogene, Netherlands and the resulting alignment of the 16S rRNA was compared for homology in the NCBI database by BLASTn nucleotide tool analysis.

Two methods were used in the bioconversion studies of ferulic acid to vanillin and vanillic acid. In the first method, fed batch, the cells were harvested in modified SM (1 g/L yeast extract was added as a sole carbon source) and ferulic acid was added when the cells were at the end of the exponential phase. In the second method, resting cell technique (Abdelkafi et al. 2008), the cells in mineral medium supplemented with 1 g/L of yeast extract were harvested at the end of the exponential phase by centrifugation, after which cells were washed and suspended in saline/phosphate buffer and then incubated aerobically, at 33 °C, in the presence of FA.

Ferulic acid, vanillin and vanillic acid in aqueous samples were analysed using high-performance liquid chromatography (HPLC) (Shimadzu) using the same protocol as Tarnawski et al. (2006). The yield of vanillic acid and vanillin production was calculated as follows: yield of vanillic acid or vanillin = (vanillic acid or vanillin concentration produced [g/L]/initial ferulic acid concentration [g/L]) × 100.

Results and discussion

Strain screening based on the bioconversion of ferulic acid to vanillic acid at high salinity

A partial 16S rRNA sequence was obtained and sequence alignment revealed that 7 out of 8 strains belong to *Halomonas* genus and 1 strain to *Marinobacter* (Table 2). Then, these 8 strains were individually examined regarding their ability to bioconvert 1 g/L ferulic acid to vanillic acid at 100 g NaCl/L. This took place at fed batch method, the cells were harvested in modified SM (1 g/L yeast extract was added as a sole carbon source) and FA was added when the cells were at the end of the exponential phase. Only the strains with closer similarity to *Halomonas elongata* were capable of bioconverting ferulic to vanillic acid after 31 h (Table 2). The highest vanillic acid at these conditions generated by strain B15 (Table 2) which corresponds to bioconversion yield of 36.5 %. However, the vanillin that was produced at these conditions (from all isolated strains) was lower than 2 mg/L. In addition, at 0 and 50 g NaCl/L the bioconversion of FA to vanillic acid from B15 was lower than 23 % (data not shown).

Phenotypic and phylogenetic characteristics of strain B15

This strain B15 is aerobic, gram-negative, motile, and it grows as short rod. Colonies on complex agar medium were 1–2 mm in diameter, smooth, circular, elevated and light yellow after 2 days. The total salts concentration for growth is 0–20 % (w/v), with an optimum at 5–8 %. Strain B15 grown at a pH range of 6–9.0 between 15–37 °C at 80 g NaCl/L. Several substrates were examined in order to define the biochemical characteristic of *Halomonas* sp. B15 such as galactose, glycerol, glucose, maltose, sucrose, tween 80, glycose, yeast extract, urea, L-histidine, L-cysteine. The isolated strain B15 has similar morphological and closely analogous biochemical characteristics to *H. elongata* except from the fact that *H. elongata* was positive in urea, negative in D-galactose (Romano et al. 1996) and it was capable of growing at anaerobic conditions (Poli et al. 2013). The isolated 16S rRNA sequence has been submitted in GenBank as *Halomonas* sp. B15 with accession number Genbank KP462856. The isolated *Halomonas* sp. B15 has been

Table 2 Isolated strains from Larnaca Salt Lake that can grow at ferulic acid (1 g/L) as a sole carbon source and produce vanillic acid at 100 g NaCl/L

Code of the Larnaca Salt isolated strain	Production of vanilic acid (mg/L) from ferulic acid (1000 mg/L) after 31 h (100 g NaCl/L) (from isolated strains)	Closest similarity (16S rRNA) of the isolated Larnaca strains with other isolated strains
Strain A1	0	Strain A1 similarity with *Halomonas ventosae* strain (GenBank: KJ009480.1)
		964/965 (100 %)
Strain A2	0	Strain A2 similarity with *Halomonas* sp. BJGMM-B32 (GenBank: JQ716237.1)
		945/945 (100 %)
Strain A3	0	Strain A3 similarity with *Marinobacter* sp. ME108 (GenBank: AJ302707.1)
		898/902 (99 %)
Strain B15 identified as *Halomonas* sp. B15 with Genbank accession number KP462856	365	Strain B15 similarity with *Halomonas elongata* DSM 2581 (GenBank: FN869568)
		1126/1128 (99 %)
Strain C1	0	Strain C1 similarity with *Halomonas caseinilytica* strain AJ261 (GenBank: FR749914.1)
		867/867 (100 %)
Strain C2	342	Strain C2 similarity with *Halomonas elongata strain NY-5 16S* (GenBank: JN903899)
		960/961 (99 %)
Strain C3	349	Strain C3 similarity with *Halomonas elongata DSM 2581* (GenBank: FN869568.1)
		960/961 (99 %)
Strain C4	308	Strain C4 similarity with *Halomonas elongata DSM 2581* (GenBank: FN869568)
		980/980 (100 %)

The similarity (16S rRNA) of the isolated strains with other isolated strain is also shown

preserved the Environmental Microbial Culture Collection at the Cyprus University of Technology.

Bioconversion of ferulic acid to vanillic acid and vanillin by resting cells of *Halomonas* sp. B15

In addition to the fed batch method, the resting cell method was used as a strategy to increase the bioconversion of ferulic acid to vanillic acid or vanillin. In this method, isolated *Halomonas* sp. B15 was used (5 g/L biomass) at 1.5 g/L ferulic acid and salinity concentrations of 0, 50, 100 and 200 g NaCl/L were tested. Vanillic acid (390 mg/L) was generated after 112 h only at 0 g NaCl/L whereas, at 50 and 100 g/L NaCl the vanillic acid was about 200 mg/L and at 200 g NaCl/L no vanillic acid was produced. Therefore, *Halomonas* sp. B15 was further tested using resting cell method (5 g/L) at lower initial FA concentrations (1.2 and 0.5 g/L at no salt). At 1.2 g/L FA the bioconverted vanillic acid was 271 mg/L after 48 h (Fig. 1a). Under these conditions, at 120 h vanillin was generated (74 mg/L) and FA was gradually consumed (Fig. 1a). At 0.5 g/L ferulic acid the highest production of vanillin (245 mg/L) took place after 48 h and decreased gradually thereafter (Fig. 1b). The vanillic acid reached its maximum value (112 mg/L) after 48 h as well as vanillin. The generation of high vanillin by *Halomonas* sp. B15 only at 0.5 g/L FA and not at 1.2 g/L FA could be due to toxicity of vanillin to cells at high concentrations of vanillin (Graf and Altenbuchner 2014).

The current study shows the ability of *Halomonas* sp. to produce vanillin at low salinity when 0.5 g/L of FA was used as a substrate. Strain B15 is the first reported

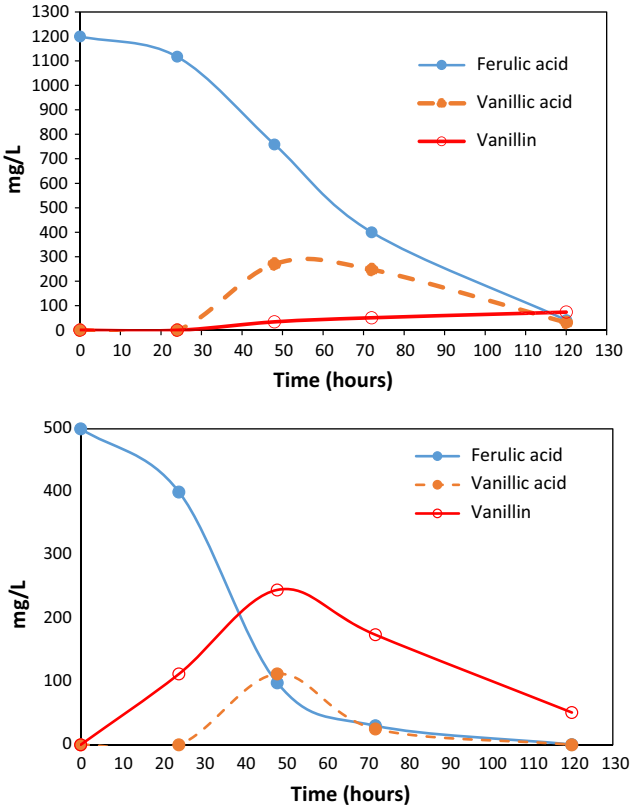

Fig. 1 **a** Ferulic (1.2 g/L) bioconversion to vanillin and vanillic acid by *Halomonas* sp. B15 at low salinity using the resting cell technique (5 g/L biomass). Bioconversion was carried out at 33 °C for 120 h with shaking at 150 rpm in 250-ml flasks containing 50 ml of saline phosphate buffer (pH 7.0). Results represent the means of two separate experiments. **b** Ferulic (0.5 g/L) bioconversion to vanillin and vanillic acid by *Halomonas* sp. B15 at low salinity using the resting cell technique (5 g/L biomass). Bioconversion was carried out at 33 °C for 120 h with shaking at 150 rpm in 250-ml flasks containing 50 ml of saline phosphate buffer (pH 7.0). Results represent the means of two separate experiments

Halomonas species that can generate a high yield of vanillin from ferulic acid at no salt. Higher yield of vanillin could be achieved in future studies by optimizing the process or by genetically modifying *Halomonas* sp. B15 strain.

Conclusion

Halomonas sp. *B15*, at fed batch method, produced vanillic acid (365 mg/L) at 100 g NaCl/L after 31 h which corresponds to ferulic acid bioconversion yield of 36.5 %.

Halomonas sp. B15 was harvested using the resting cell method at 0.5 g/L ferulic acid with low salinity and this resulted in the highest production of vanillin (245 mg/L) after 48 h, which corresponds to bioconversion yield of 49 %. *Strain* B15 is the first reported *Halomonas* species that can generate a high yield of vanillin from ferulic acid.

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