New BIOTECHNOLOGY xxx (2017) xxx-xxx

Contents lists available at ScienceDirect

New BIOTECHNOLOGY

journal homepage: www.elsevier.com

Full length article

Fed-batch production of vanillin by *Bacillus aryabhattai* BA03

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ARTICLE INFO

Article history:
Received 1 November 2016
Received in revised form 17 July 2017
Accepted 30 July 2017
Available online xxx

Keywords:
Bacillus aryabhattai
Ferulic acid
Vanillin
4-Vynilguaiacol
Decarboxylation

ABSTRACT

Bacillus aryabhattai BA03, a strain isolated in our laboratory, has interesting properties related to the production of natural aromas and flavors. Specifically, we have found that it was able to produce vanillin from ferulic acid (FA). Furthermore, this strain produces high amounts of 4-vinylguaiacol in only 14 h, this being the only intermediate metabolite observed in the process. FA is an inexpensive feedstock for the production of natural value-added compounds when extracted from lignocellulosic wastes. In this study, we optimized the operational conditions (temperature, pH and agitation), medium composition and bioconversion technology (batch or fed-batch) to produce vanillin. In a fed-batch process conducted with just one additional supplementation after 24 h, the maximal concentration of vanillin (147.1 ± 0.9 mg/L) was observed after 216 h (Q_V = 0.681 mg/L h; $Y_{V/FA}$ = 0.082 mg/mg) after degrading 90.3% FA. In view of our data, we postulate that *Bacillus aryabhattai* BA03 carries out a decarboxylation of ferulic acid as a metabolic pathway.

Introduction

Bacillus is a wide genus with extensive diversity of physiological and metabolic abilities. A number of studies have reported on the testing of free cells of *Bacillus* spp. or their enzymes in the metabolization of environmental pollutants, and in the biotransformation of inexpensive or natural substrates into high-value compounds. This genus is able to produce an extensive variety of compounds such as biopolymers [1,2], biosurfactants [3,4], bacteriocins [5,6] and bio-hydrogen [7] among others. In addition, it can be applied as a biopesticide [8,9] and in biodegradation of hazardous compounds [10,11].

Previous research in our laboratory led to the isolation, from contaminated cryovials, of a scarcely studied *Bacillus* sp., later identified as *Bacillus aryabhattai* BA03. Thus far, this species has only been used to produce asparaginases [12] and poly(3-hydroxybutyrate) [13], to degrade some recalcitrant compounds like organophosphate insecticide [14], and for the bioremediation of heavy metals [15]; it has not been used to produce natural aromas or flavors. Preliminary experiments showed that is able to produce natural value-added compounds such as vanillin and 4- vynilguaiacol (denoted 4VG) [16]. However, few species have so far been proposed to produce these compounds with this genus. For instance, Chen et al. [17] only produced 0.16 mg/L vanillin using *Bacillus subtilis* B7-S, and Karmakar et al. [18] assayed a newly isolated strain of *Bacillus coagulans* to enhance the vanillin production up to 16.8 mg/L from 1 g/L ferulic acid (FA). On the other hand, Lee et al. [19] developed an aqueous-organic solvent two-phase systems to produce 1.5 g/L of 4VG using *Bacillus pumilus*.

Ferulic acid, a phenolic compound found in the cell wall of lignocellulosic materials, is an economically viable resource if obtained from agricultural or brewery waste, where it is covalently linked with a variety of carbohydrates such as a glycoside conjugate, ester or amide, with special treatments necessary to release it [20,21]. FA needs to be extracted from the lignocellulosic material using a variety of conventional physical, chemicals and physicochemical treatments [21], or non-conventional techniques such as microwave-assisted extraction, ultrasound-assisted extraction, etc. [22]. One of their applications is in the production of natural value-added compounds such as vanillin and 4-vynilguaiacol [20]. In this study, we tested the ability of *Bacillus aryabhattai* BA03 to produce vanillin and intermediate compounds, such as 4VG, from synthetic FA.

Materials and methods

Chemicals

All chemicals, including methanol for HPLC, were of analytical grade and purchased from Sigma–Aldrich (St Louis, MO), and all culture media components were supplied by Panreac Química SAU (Barcelona, Spain).

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https://doi.org/10.1016/j.nbt.2017.07.012
1871-6784/

Microorganism and inoculum preparation

Bacillus aryabhattai BA03 was previously isolated and characterized in our laboratory [16], and stored in cryovials with 20% (v/v) glycerol solution at −20 °C until use. The strain has been deposited in the Spanish Type Culture Collection (CECT) as *Bacillus aryabhattai* strain BA03 (accession number CECT 9010). The sequence data to the European Molecular Biology Laboratory (EMBL) and Genbank databases has the accession number LN824023.

Bacterial reconstitution and growth was carried out in 250 mL Erlenmeyers flasks containing 50 mL of Trypticasein Soy Broth (TSB) placed in an orbital shaker (Optic Ivymen System, Comecta S.A., distributed by Scharlab, Madrid, Spain) at 37 °C and 150 rpm. TSB contains (per liter) a pancreatic digest of casein 17 g, glucose monohydrate 2.5 g, sodium chloride 5 g, dipotassium phosphate 2.5 g and a papaic digest of soy bean 3 g. After 24 h, cells were centrifuged and cleaned twice with phosphate buffered saline (PBS). Five mL was used to measure the inoculum concentration through oven-drying at 105 °C (Binder-Model 53 ED, Tuttlingen, Germany) to a constant weight.

Biotransformation of ferulic acid

The biotransformation process was carried out using three media: Nutrient broth (NA) consisting of peptone 5 g, NaCl 5 g, yeast extract 2 g and beef extract 1 g (per liter); TSB as previously described, and a minimal medium (YE) with only 5 or 10 g yeast extract per liter. Some modifications were also assayed, as described in Table 1. Agar media plates had the same composition after addition of 20 g/L of agar.

All media were autoclaved (Trade Raypa SL, Terrassa, Barcelona, Spain) at 121 °C for 20 min and supplemented with the required volume of stock filter-sterilized solution of 50 g/L FA dissolved in 0.5 M NaOH in order to achieve an initial FA concentration of 1 or 4.5 g/L. pH (pHmeter BASIC 20+, CRISOL, Alella, Barcelona Spain) was then adjusted to 5, 7 or 8.5 with sterilized solutions of HCl or 0.5 M NaOH. Culture media were subsequently dispensed into 250 mL Erlenmeyer flasks with a final volume of 100 mL. Flasks were inoculated and incubated at different temperatures (30, 35, 37 or 40 °C) and shaking speeds (100, 150 or 200 rpm). FA was directly supplemented at the beginning of the biotransformation (batch technology) or intermittently (fed-batch technology) according to the conditions (number of additional supplementations and the intervals between them) as described in Table 1. Tests were performed in triplicate and control flasks were cultured without inoculum to test for the effect of the abiotic parameters.

Analytical methods

Samples (1.5 mL) were taken at determined times to measure pH and growth by optical density at 600 nm in a UV–vis Spectrophotometer (Libra S60-Biochrom, Cambridge, U.K.). Samples were then filtered through 0.2 μm pore membranes (Sartorius, Goettingen, Germany) in order to analyze the concentrations of FA, vanillin, 4VG, vanillic acid and vanillyl alcohol in the liquid phase, using a reverse phase HPLC system (Agilent model 1200, Palo Alto, CA, USA) with UV-diode array detector and 4.6 × 150 mm Zorbax SB-Aq column (Agilent, Palo Alto, CA, USA). The elution program was carried out at 35 °C. The mobile phase at a flow rate of 1 mL/min comprised a mixture of two solvents, a 2.5% formic acid aqueous solution (v/v) and B 100% methanol. The following elution profile was used: solvent A started at 100% holding for 35 min, then decreased to 52% at 5 min, continued using only solvent B at 16 min, returned to 100% of A at 4 min, and the column was re-equilibrated for 5 min before the next injection. Elute was continuously monitored by UV detector at 276 nm.

Sugars (glucose), alcohols (ethanol) and organics compounds (acetic and citric acids) were also measured by HPLC (Agilent model 1200, Palo Alto, CA) equipped with a refractive index detector and an Aminex HPX-87H ion exclusion column (Bio Rad 300 mm × 7.8 mm, 9 m particles). Elution program with 0.003 M sulfuric acid was at a flow rate of 0.6 mL/min at 50 °C for 23 min.

Standards solutions of vanillic acid, vanillyl alcohol, 4VG, ethanol, acetic acid and citric acid for HPLC analysis were prepared at maximal concentrations of 1 g/L. Vanillin was prepared at 0.3 g/L, FA at 5 g/L and glucose at 3 g/L.

Statistical methods

In order to determine the most suitable operational conditions, statistical analyses were submitted to analysis of variance (ANOVA) by the Statistica Software 13.0 (Statsoft, Tulsa, OK, USA). They were compared using the Tukey's test at significance level $P < 0.05$, and different letters were used to label values with statistically significant differences.

Results and discussion

Influence of operational conditions (temperature, pH and agitation)

Preliminary experiments were conducted to evaluate the operational conditions on FA biotransformation. The effects of temperature, pH and agitation were studied using AN as the culture medium. 4VG and vanillin were the only phenolic compounds obtained in considerable amounts. Table 1 summarizes the maximal concentrations and significance of both extracellular metabolites, as well as some of the parameters calculated for the process. Additionally, control experiments starting with 1 g/L FA under the same experimental conditions confirmed that FA was not abiotically transformed.

A positive correlation between temperature and vanillin production was observed from 30 to 37 °C (experiments 1–3 in Table 1), where a maximal amount of 37.7 ± 0.26 mg/L was obtained, corresponding to a global volumetric productivity (Q_V) of 0.083 mg/L h and a product yield ($Y_{V/FA}$) of 0.041 mg/mg. However, an increment up to 40 °C (experiment 4) served to decrease this value slightly (32.5 ± 0.01 mg/L, Q_V = 0.071 mg/L h; $Y_{V/FA}$ = 0.036 mg/mg). Significant differences ($P < 0.05$) were found at 37 and 30 °C, but not at 35 and 40 °C. Consequently, 37 °C was chosen for further experimentation. The low values calculated for the global volumetric productivity can be attributed to both the relatively low level of vanillin generated and the prolonged times required to achieve these values (456 h). At this time, FA was degraded by more than 90% in all cases except under 30 °C (84.7%), representing global rates of FA degradation (Q_{FA}) of 1.851–2.013 mg/L h.

The analysis of these data also indicates a rapid initial depletion of FA with a transitory peak of accumulation of 4VG in only 14 h (Table 1). Therefore, significant differences ($P < 0.05$) were found when the temperature increased from 30 to 40 °C since the concentration of 4VG increased from 305.7 ± 33.8 up to 510.0 ± 21.5 mg/L, respectively. This indicates an 11 to 15.7-fold higher amount of 4VG in terms of vanillin. The increases in the parameters calculated was also considerable in ~~that~~ the global volumetric productivity (Q_{4VG}) at 40 °C was 36.428 mg/L h, the global rate of FA degradation (Q_{FA})

Table 1
Data summarizing the influence of the operational conditions and medium composition on ferulic acid degradation by *Bacillus aryabhattai* in batch systems.

Exp.	Medium	T	Agit.	pH	$FA_{t=0}$	t	$4VG_{max}$	Q_{4VG}	FA	FA_d	Q_{FA}	$Y_{4VG/FA}$	t	V	Q_V	FA	FA_d	Q_{FA}	$Y_{V/FA}$	4VG/V
		(°C)	(rpm)		(mg/L)	(h)	(mg/L)	(mg/L h)	(mg/L)	(%)	(mg/L h)	(mg/mg)	(h)	(mg/L)	(mg/L h)	(mg/L)	(%)	(mg/L h)	(mg/mg)	
a) Influence of operational conditions																				
1	NA	30	150	7	997.3 ± 1.1	14	305.7 ± 33.8[a]	21.836	331.6 ± 19.0	66.8	47.552	0.46	456	24.4 ± 0.76[a]	0.053	153.1 ± 10.6	84.7	1.851	0.029	12.5
2	NA	35	150	7	997.3 ± 1.1	14	421.2 ± 29.2[b]	30.086	136.1 ± 6.8	86.4	61.519	0.49	456	29.4 ± 0.35[b]	0.064	79.6 ± 6.8	92.0	2.013	0.032	14.3
3	NA	37	150	7	997.3 ± 1.1	14	414.5 ± 27.8[b]	29.610	121.8 ± 2.0	87.8	62.540	0.47	456	37.7 ± 0.26[c]	0.083	89.1 ± 0.78	91.1	1.992	0.041	11.0
4	NA	40	150	7	997.3 ± 1.1	14	510.0 ± 21.5[c]	36.428	101.3 ± 4.1	89.8	64.003	0.57	456	32.5 ± 0.0[b]	0.071	90.03 ± 1.3	91.0	1.990	0.036	15.7
5	NA	37	150	5	990.9 ± 1.1	14	0.0 ± 0.0[a]	0.000	990.0 ± 2.1	0.1	0.061	0.00	432	0.0 ± 0.0[a]	0.000	990.9 ± 1.1	0.0	0.000	0.000	–
6	NA	37	150	7	997.3 ± 1.1	14	414.5 ± 27.8[b]	29.610	121.8 ± 2.0	87.8	62.540	0.47	456	37.7 ± 0.26[b]	0.083	89.1 ± 0.78	91.1	1.992	0.041	11.0
7	NA	37	150	8.5	991.8 ± 1.1	14	453.0 ± 24.3[b]	32.355	174.1 ± 8.2	82.4	58.407	0.55	432	46.6 ± 0.49[c]	0.108	42.1 ± 1.1	95.8	2.198	0.049	9.7
8	NA	37	100	8.5	995.1 ± 1.0	14	430.4 ± 14.8[a]	30.746	146.6 ± 7.1	85.3	60.608	0.51	408	38.7 ± 0.44[a]	0.095	120.4 ± 15.4	87.9	2.144	0.044	11.1
9	NA	37	150	8.5	991.8 ± 1.1	14	453.0 ± 24.3[a]	32.355	174.1 ± 8.2	82.4	58.407	0.55	432	46.6 ± 0.49[a,b]	0.108	42.1 ± 1.1	95.8	2.198	0.049	9.7
10	NA	37	200	8.5	995.1 ± 1.0	14	455.9 ± 3.1[a]	32.562	184.1 ± 4.9	81.5	57.925	0.56	408	45.5 ± 0.33[b]	0.112	54.2 ± 4.2	94.6	2.306	0.048	10.0
b) Influence of medium composition																				
11	NA	37	150	8.5	991.8 ± 1.1	14	453.0 ± 24.3	32.355	174.1 ± 8.2	82.4	58.407	0.55	432	46.6 ± 0.49	0.108	42.1 ± 1.1	95.8	2.198	0.049	9.7
12	NA	37	150	8.5	4895.6 ± 5.6	14	848.6 ± 0.7	60.611	2527.9 ± 2.7	48.4	169.122	0.36	96	52.1 ± 1.0	0.543	1939.9 ± 8.9	60.4	30.789	0.018	16.3
13	TBS	37	150	8.5	996.1 ± 1.0	14	187.2 ± 17.6	13.371	41.0 ± 3.2	95.9	68.224	0.20	168	21.1 ± 0.49	0.125	22.1 ± 0.34	97.8	5.798	0.022	8.9
14	TBS	37	150	8.5	4662.4 ± 7.8	14	901.2 ± 45.3	64.371	1581.5 ± 10.9	66.1	220.061	0.29	168	46.5 ± 1.8	0.277	963.6 ± 39.7	79.3	22.016	0.013	19.4
15	NA – Salt	37	150	8.5	4401.0 ± 40.3	14	290.0 ± 12.0	20.714	2482.2 ± 17.2	43.6	137.061	0.15	120	47.8 ± 0.08	0.398	1499.3 ± 77.3	65.9	24.181	0.016	6.1
16	NA *2	37	150	8.5	4557.0 ± 78.3	14	944.3 ± 0.6	67.450	2179.5 ± 3.3	52.2	169.820	0.40	120	56.6 ± 1.7	0.472	1617.8 ± 8.1	64.5	24.493	0.019	16.7
17	YE 5 g/L	37	150	8.5	996.1 ± 1.0	14	345.1 ± 31.3	24.651	103.5 ± 5.3	89.6	63.758	0.39	192	41.4 ± 0.0	0.216	27.8 ± 0.31	97.2	5.043	0.043	8.3
18	YE 10 g/L	37	150	8.5	996.1 ± 1.0	14	498.7 ± 28.2	35.621	24.9 ± 3.5	97.5	69.374	0.51	192	45.8 ± 0.69	0.238	19.5 ± 1.3	98.0	5.087	0.047	10.9
19	YE 10 g/L	37	150	8.5	4560.3 ± 9.0	14	999.9 ± 36.5	71.425	2068.1 ± 11.5	54.6	178.014	0.40	216	138.3 ± 1.9	0.640	1487.0 ± 43.9	67.4	14.228	0.045	7.2

T: Temperature; Agit.: Agitation; $FA_{t=0}$: ferulic acid at the beginning of the process; t: time; $4VG_{max}$: maximal amount of 4VG achieved at time t; FA: residual amount of FA at time t; Q_{4VG}: global volumetric productivity of 4VG; FA_d: percentage of FA degradation; Q_{FA}: global rate of FA degradation; $Y_{4VG/FA}$: 4VG yield; V: maximal amount of vanillin achieved at time t; Q_V: global volumetric productivity of vanillin; $Y_{V/FA}$: vanillin yield.
NA: nutrient broth; TBS: trypticasein soy broth medium; YE: yeast extract medium.
Different letters indicate statistically significant differences among values in the same section of operational conditions ($P < 0.05$).

was 64.003 mg/L h, and the product yield ($Y_{4VG/FA}$) was 0.57 mg/mg. However, this peak was continuously degraded and no amount of 4VG was quantified at the time in which vanillin reached the highest point (456 h).

The biotransformation was affected more by pH (experiments 5–7) since FA was not degraded at all under pH 5, and consequently neither 4VG nor vanillin were observed (Table 1). This finding is in agreement with our previously study [16] in which it was noted that this strain was unable to transform FA into vanillin in acid media. However, the increase of pH showed positive effects on vanillin production achieving a maximal amount of 46.6 ± 0.49 mg/L (Q_V = 0.108 mg/L h; Q_{FA} = 2.198 mg/L h; $Y_{V/FA}$ = 0.049 mg/mg) at pH 8.5, and therefore significant differences were obtained ($P < 0.05$) increasing the values in respect of the previous experiments performed at pH 7. Previous studies showed that using FA based culture media, *Bacillus aryabhattai* BA03 first made alkaline substances which increased the pH up to 8–8.5 [16]. Consequently, the use of a basic medium avoids this stage, thus facilitating the metabolism of FA degradation. As a result, a pH value of 8.5 was chosen for the following trials. In addition, 4VG also experienced a slight increment up to 453.0 ± 24.3 mg/L (Q_{4VG} = 32.355 mg/L h; Q_{FA} = 58.407 mg/L h; $Y_{4VG/FA}$ = 0.55 mg/mg).

Finally, the influence of agitation was negligible (experiments 8–10 in Table 1) since the vanillin and 4VG concentrations hardly oscillated in the interval studied (100–200 rpm). No significant differences were found between 100 and 150 rpm, or 150 and 200 rpm ($P > 0.05$). An intermediate value (150 rpm) was thus established to continue the study.

Medium composition for biotransformation of ferulic acid

Previous studies [16] have concluded that culture media containing glucose did not allow vanillin production. Some authors have suggested that bacilli have two regulatory metabolic mechanisms: induction and carbon catabolite repression (CCR), and thus that catabolic enzymes are only synthesized when their substrate is present in the growth medium (induction) and/or preferred carbon and energy sources are absent (CCR) [23,24]. Consequently, the operational parameters were optimized (37 °C, pH = 8.5 and 150 rpm) using a medium formulated in the absence of sugars (NA medium), with the exception of inoculum preparation, where TSB was preferred in order to achieve high amounts of biomass in a shorter period.

In order to improve the amounts of vanillin, the influence of medium composition was addressed; Table 1 sets out all the trials assayed (experiments 11–19). Hence, notwithstanding possible CCR, TSB medium (containing 2.5 g/L glucose) was studied to confirm our previous findings. In addition, other conditions were selected including NA medium without salts (sodium chloride), using twice the amount of NA medium, or using only a nitrogen source (yeast extract). Finally, some experiments were conducted increasing the initial amount of FA by 4.5 fold. Previously we tested on plates the toxicity of FA on the microorganism (data not shown), finding that it was able to support concentrations of up to 10 g/L.

The results confirm that this strain prefers the absence of sugars, since experiments using an NA medium (experiments 11 and 12) produced higher amounts of vanillin than experiments using culture media containing sugars (experiments 13 and 14). Starting with 1 g/L FA the amount of vanillin produced in experiment 11 (46.6 ± 0.49 mg/L; $Y_{V/FA}$ = 0.049 mg/mg) doubled the value achieved in experiment 13 (21.1 ± 0.49 mg/L; $Y_{V/FA}$ = 0.022 mg/mg), showing the detrimental influence of glucose. The only positive effect in either experiment was the considerable reduction in the time to achieve these values, which was reduced from 432 to 168 h, thus increasing the kinetic parameters from (Q_V = 0.108 mg/L h; Q_{FA} = 2.198 mg/L h) to (Q_V = 0.125 mg/L h; Q_{FA} = 5.798 mg/L h) respectively.

Working at the higher amount of initial FA, it could also be shown that the strain supports a wide range of salinities by comparing the results of experiments 12 (intermediate value of salinity), 15 (no salinity) and 16 (higher amount of salinity). The results indicated that although *Bacillus aryabhattai* BA03 can produce 47.8 ± 0.08 mg/L vanillin in the absence of salts, the results can be improved up to 52.1 ± 1.0–56.6 ± 1.69 mg/L in media containing salt.

Nevertheless, the most notable finding was that the best results were achieved in the presence of yeast extract (10 g/L) as the only nitrogen source, thus reducing considerably the costs of formulating the appropriate culture media, with 138.3 ± 1.49 mg/L vanillin obtained in 216 h (Q_V = 0.640 mg/L h; Q_{FA} = 14.228 mg/L h; $Y_{V/FA}$ = 0.045 mg/mg). We therefore concluded that *Bacillus aryabhattai* BA03 is highly dependent on the nitrogen source, which is in agreement with Bergey's [25] manual, in which it was shown that many bacilli do not require growth factors, but that yeast extract often stimulates growth.

Regarding 4VG production, we found that independently of the culture medium employed (NA or YE), the results and performance were similar. The production of 4VG in NA and YE media from 1 g/L of FA by *Bacillus aryabhattai* BA03 under optimal operational conditions (37 °C, 150 rpm and pH = 8.5) is illustrated in Fig. 1. In around 11 h, *Bacillus aryabhattai* BA03 consumed most of the 1 g/L of FA to produce 4VG. This production began after 2–4 h, depending on the culture medium, achieving a maximal value in approximately 12–14 h. Although production in NA medium was faster in the initial stage, after 12 h similar conditions were achieved in both media. Hence, by using 10 g/L YE and 1 g/L FA (experiment 18), 498.7 ± 28.2 mg/L (Q_{4VG} = 35.621 mg/L h; Q_{FA} = 69.374 mg/L h; $Y_{4VG/FA}$ = 0.51 mg/mg) were obtained, similar to the 453.0 ± 24.3 mg/L (Q_{4VG} = 32.355 mg/L h; Q_{FA} = 58.407 mg/L h; $Y_{4VG/FA}$ = 0.55 mg/mg) achieved in the NA medium (experiment 11). After that threshold, 4VG was gradually degraded and only after prolonged times of cultivation did vanillin start to be visible. When we increased the initial FA concentration 4.5-fold, the amount of 4VG generated by *Bacillus aryabhattai* BA03 also increased (see Table 1), attaining a maximal value of 999.9 ± 36.5 mg/L (Q_{4VG} = 71.425 mg/L h; Q_{FA} = 178.014 mg/L h; $Y_{4VG/FA}$ = 0.40 mg/mg) in the final assay (experiment 19).

Influence of bioconversion technology (batch or fed-batch) in ferulic acid conversion

Finally, considering the positive effect of increasing 4.5-fold the initial amount of FA (experiments 18 and 19) we investigated the influence of the fed-batch technology on the bioconversion of FA into vanillin. First, the initial FA was supplemented with 1 g/L FA 4 additional times at 3 different intervals (6, 14 or 24 h) to reach a total concentration of 4.5–5 g/L FA (experiments 20–22 in Table 2). The intervals were selected on the basis of the following considerations: a) the growth curve of *Bacillus aryabhattai* BA03 (data not shown) showed that the exponential phase of the cell cycle was completed at 6 h; b) 4VG reached maximal concentrations after 14 h in previous batch experiments (see data of Table 1 and Fig. 1); and c) 4VG was consumed swiftly after 24 h while FA degradation was slowed down (data not show). The results confirmed the importance of expanding the time of supplementation in order to avoid an accumulation of FA; the best results were achieved in experiment 22 when the interval of supplementation (24 h) allowed a complete depletion of FA, although

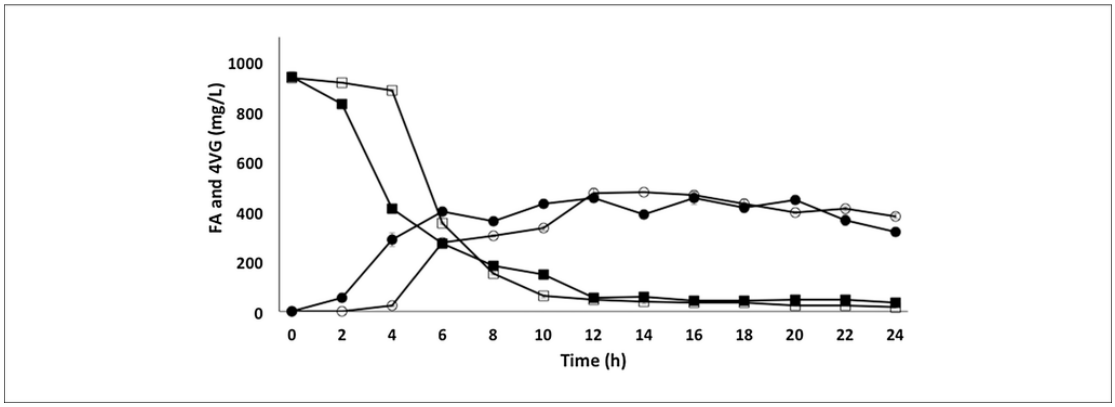

Fig. 1. 4VG production in NA and YE media from 1 g/L of FA by Bacillus aryabhattai under optimal operational conditions (37 °C, 150 rpm and pH = 8.5). Ferulic acid in NA medium (■), Ferulic acid in YE medium (?), 4VG in NA medium (●), 4VG in YE medium (○). Ferulic acid in NA medium (Dotted line), Ferulic acid in YE medium (Dashed line), 4VG in NA medium (Straight line), 4VG in YE medium (Dot and hyphen line).

Table 2
Bioconversion of ferulic acid by *Bacillus aryabhattai* BA03 in fed-batch systems using yeast extract (10 g/L) as a culture medium. Experiments performed at 37 °C, 150 rpm and pH = 8.5.

Exp.	tS	N° S	$FA_{t=0}$	t	V	Q_V	FA	FA_d	Q_{FA}	$Y_{V/FA}$
	(h)		(mg/L)	(h)	(mg/L)	(mg/L h)	(mg/L)	(%)	(mg/L h)	(mg/mg)
20	6h	4	983.3 ± 0.67	144	40.9 ± 1.9	0.284	1296.7 ± 13.1	73.6	25.137	0.011
21	14h	4	983.3 ± 0.67	144	49.0 ± 0.8	0.341	1707.8 ± 51.1	65.3	22.282	0.015
22	24h	4	983.3 ± 0.67	144	73.4 ± 0.8	0.510	1938.0 ± 9.8	60.6	20.684	0.025
23	14h	2	993.4 ± 0.59	192	62.4 ± 0.7	0.325	630.9 ± 6.3	78.8	12.237	0.027
24	24h	2	993.4 ± 0.59	168	62.8 ± 0.8	0.374	694.6 ± 4.0	76.7	13.606	0.027
25	24h	1	990.0 ± 0.49	216	147.1 ± 0.9	0.681	193.0 ± 2.7	90.3	8.273	0.082

tS: time between each supplementation; N° S: number of supplementation in each experiment; $FA_{t=0}$: FA at the beginning of the process; t: time; V: maximal amount of vanillin achieved at time t; Q_V: global volumetric productivity of vanillin; FA: residual amount of FA at time t; FA_d: percentage of FA degradation; Q_{FA}: global rate of FA degradation; $Y_{V/FA}$: vanillin yield.

1938 ± 9.8 mg/L FA still remained after 144 h (39.4%) with a Q_{FA} = 20.684 mg/L h. Nevertheless, vanillin achieved a maximum concentration of only 73.4 ± 0.81 mg/L (Q_V = 0.510 mg/L h; $Y_{V/FA}$ = 0.025 mg/mg). Using this technology, and considering the fast generation of 4VG and the continuous supplementation of FA, it is not possible to observe a maximal production of 4VG, since this compound is generated and consumed continuously.

Two additional experiments (23 and 24) were conducted subsequently, reducing the number of additional supplementations from 4 to 2 (see Table 2) in order to minimize the final untreated concentration of FA in the culture broth. Nevertheless, the vanillin concentration was slightly reduced, and 694.6 ± 4.0 mg/L FA still remained at the end of the cultivation. Accordingly, a final fed-batch process was carried out with just one additional supplementation after 24 h. Fig. 2 shows the course with time for FA degradation and 4VG and vanillin formation, and Table 2 illustrates that under these experimental conditions the maximal amount of vanillin (147.1 ± 0.9 mg/L) was observed after 216 h (Q_V = 0.681 mg/L h; $Y_{V/FA}$ = 0.082 mg/mg). More

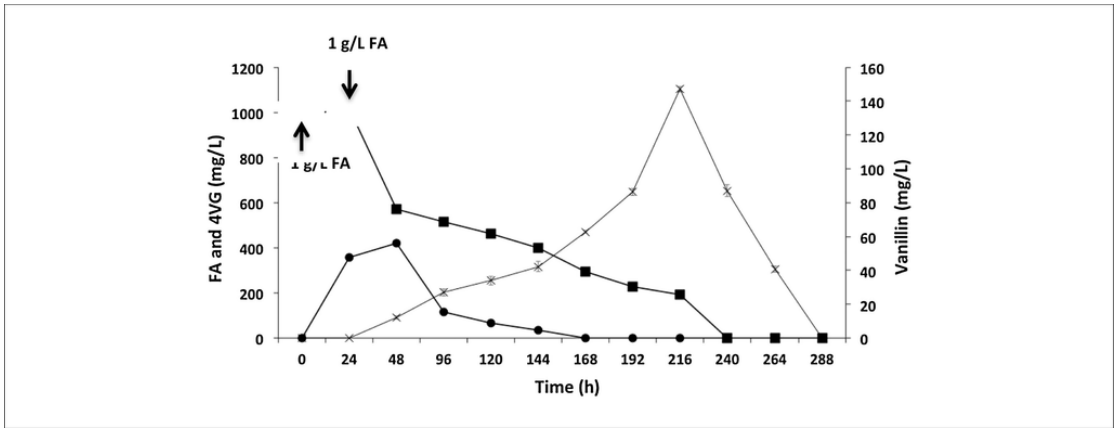

Fig. 2. Fed-batch bioconversion of ferulic acid by *Bacillus aryabhattai* (experiment 25). Ferulic acid (1 g/L) was added at time 0 and after 24 h. Ferulic acid (■), 4 VG (●), vanillin (×). Ferulic acid (Straight line), 4 VG (Dashed line), vanillin (Dotted line). Arrows indicated the interval of supplementation with 1 g/L of Ferulic acid.

over, the remaining FA was only 193.0 ± 2.7 mg/L, meaning that 90.3% was degraded (Q_{FA} = 8.273 mg/L h). Consequently, in spite of *Bacillus aryabhattai* BA03 is able to support higher concentrations of FA (as described upwards), we concluded that this strain better assimilates the compound when it is supplemented at different intervals (fed-batch systems), producing higher levels of vanillin in comparison with results of others [17,18].

In addition, we confirmed that this strain produces only 4VG as the unique intermediate compound. Rosazza et al. [26] have explained that, depending on the microorganism, the biochemical pathway employed to transform FA into vanillin is different. Priefert et al. [27] indicated that genus *Bacillus* could biotransform FA by decarboxylation into 4VG, or by Coenzyme-A-independent deacetylation, and directly generate vanillin. According to our data, *Bacillus aryabhattai* BA03 successfully performs a decarboxylation of FA.

Conclusions

Bacillus aryabhattai BA03 is a halotolerant strain able to grow under a wide range of conditions, although the production of natural vanillin can be optimized under specific operational conditions (37 °C and 150 rpm). Furthermore, the strain showed distinctive features of extremophile microorganisms, preferring alkaline media to produce vanillin (pH 8.5). In addition, this strain was shown to be highly dependent on a nitrogen source, and better results were obtained in culture media formulated with FA-based media supplemented exclusively by yeast extract. Regarding the influence of bioconversion technology, a fed-batch process carried out with just one additional supplementation of FA after 24 h was the most suitable system. Finally, the fast production of 4-vinylguaiacol as the unique intermediate compound was observed. This metabolite was generated under a variety of conditions and quickly degraded after 24 h, resulting in a culture broth containing vanillin as the sole product after prolonged times of cultivation.

Acknowledgements

We are grateful to the Spanish Ministry of Economy and Competitiveness for financial support given for this study (project CTQ2015-71436-C2-1-R), which has partial financial support from the FEDER funds of the European Union, and to the Spanish Ministry of Education, Culture and Sports for Alicia Pérez Paz's FPI.

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