Bio-based Chemical Production from *Arundo donax* Feedstock Fermentation using *Cosenzaea myxofaciens* BPM1

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Bio-based organic acids are an eco-friendly alternative to petroleum-derived products. In this work, the production of organic chemicals was investigated for the first time in the *Cosenzaea myxofaciens* species using hydrolysed lignocellulosic biomass from *Arundo donax*. The strain *C. myxofaciens* BPM1, isolated from bovine rumen, was able to produce a high amount of lactic acid, followed by acetic and succinic acids in synthetic substrate in microaerophilic and anaerobic conditions. When hydrolysed lignocellulosic biomass from *Arundo donax* supplemented with several nitrogen sources was used as substrate in separate hydrolysis and fermentation in anaerobic conditions, a significant increase in organic acids was recovered, reaching values up to 12.13 ± 0.17, 1.68 ± 0.1, and 5.23 ± 0.04 g L^{-1} of lactate, succinate, and acetate, respectively. Moreover, the strain *C. myxofaciens* BPM1 was capable of synthesizing a small amount of ethanol, with a resulting concentration ranging from 0.67 ± 0.05 to 1.46 ± 0.03 g L^{-1}. This work shows that the strain *C. myxofaciens* BPM1 is a potential source of interesting bio-based chemicals for a wide range of industrial applications. In addition, the inexpensive fermentation process using *A. donax* hydrolysate and corn steep liquor as carbon and nitrogen sources could be suitable for economical and efficient production of succinic acid in industrial processes.

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INTRODUCTION

Organic chemicals, fuels, and polymers are currently mainly derived from petroleum-based feedstocks (de Jong *et al*. 2012). Among these, organic acids such as lactate, succinate, and acetate are a key group of building-block chemicals. They are precursors for many important chemicals that are used for a broad range of industrial applications such as solvents, biodegradable plastics, pharmaceuticals, polymers, and food (Taskila and Ojamo 2013; Akhtar *et al.* 2014). To overcome issues associated with fossil-derived chemicals regarding high production cost, environmental concerns, and dependence on energy imports, there is growing interest in the synthesis of chemicals based on biological processes. In fact, most organic acids are produced as intermediates of major metabolic pathways, the tricarboxylic acid (TCA) cycle, or as final natural products of anaerobic metabolism in microbial fermentation (Sauer *et al.* 2008; Yin *et al.* 2015).

Therefore, many different bacteria, yeasts, and filamentous fungi are good candidates for natural producers of organic acid. At an industrial level, the use of bacteria offers several advantages, such as fast growth rate and clear genetic background, which makes them easily engineered (Kirchner and Tauch 2003). Because the current market for fossil-based organic acids is small due to price limitations, research that investigates the production of bio-based organic acids by microorganisms is growing quickly (Sauer *et al.* 2008). Many prokaryotic microorganisms, *e.g. Escherichia coli*, *Lactobacillus plantarum*, *Lactococcus lactis*, *Bacillus* spp., *Corynebacterium glutamicum*, *Mannheimia succiniciproducens*, *Ruminococcus albus*, *Prevotella ruminicola*, *Bacteroides amylophilus*, and *Bacteroides fragilis* isolated from rumen and other natural environments, have been assayed and engineered to evaluate and increase their ability to produce of organic acids (Donnelly *et al.* 1998; Lee *et al.* 2002; Kirchner and Tauch 2003; Solem *et al.* 2003; Bechthold *et al.* 2008; Oh *et al.* 2008; Balzer *et al.* 2013; Taskila and Ojamo 2013).

Moreover, using renewable carbon sources for microbial fermentation is the most prominent eco-technology strategy for bio-based chemical production (McKinlay *et al.* 2007). Lignocellulosic biomass is the most abundant renewable energy source on Earth, including dedicated energy crops, such as miscanthus, switchgrass, *Arundo donax*, *Populus nigra*, and *Eucalyptus camaldulensis* that can be easily grown in croplands that are not suitable for food crops because of degraded, contaminated soils or soils subjected to accelerated erosion (Fiorentino *et al.* 2010; Ventorino *et al.* 2015). The production of chemicals from hydrolysed lignocellulosic biomass is a very attractive technology because of the abundance, high productivity, resistance to biotic and abiotic stresses, and competitive cost of the raw material (Mariani *et al.* 2010; Akhtar *et al.* 2014). In fact, bio-based processes are more cost-effective than petroleum-based processes (Song and Lee 2006).

In the present work, an ecological approach was developed to isolate bacteria able to synthetize interesting organic acids from natural ecosystems. A new lactic and succinic acid-producing strain was isolated, belonging to species *Cosenzaea* (*C.*) *myxofaciens*, with an attractive substrate utilization spectrum. Moreover, second-generation bio-based chemical production was evaluated, and an inexpensive separate hydrolysis and fermentation (SHF) process using pretreated *A. donax* lignocellulosic biomass cultivated in marginal soils was developed.

EXPERIMENTAL

Isolation and Identification of Bacterial Strain BPM1

The bacterial strain BPM1 was isolated from a sample of rumen from recently slaughtered cattle. Aliquots of samples (10 g each) were placed into 200 mL of enrichment medium supplemented with ionoporic antibiotics lasalocid (16 mg L^{-1}, Sigma-Aldrich, Milan, Italy) and monensin (10 mg L^{-1}, Sigma-Aldrich) to inhibit acetic acid-producing and hydrogen-producing microorganisms as described by Lee *et al.* (2002). The cultures were incubated for 18 h at 37 °C in a 5% CO_2 atmosphere (CO_2 incubator NOVACELL CO170, Esco CelCulture Incubatori, Italy). The enrichment cultures were serial decimal diluted (until 10^{-7}) in phosphate-buffered saline solution. Each dilution was spread (100 µL) on two plates with selective solid isolation medium (SSIM) containing, per liter, 5 g of glucose (Sigma-Aldrich), 23 g of peptone (Oxoid, Milan, Italy), 5 g of yeast extract (Oxoid), 2 g of NaCl (Sigma-Aldrich), 0.4 g of $NaHCO_3$ (Sigma-Aldrich), 0.5 g of

cysteine-HCl (Sigma-Aldrich), 0.25 g of soluble pyrophosphate (Sigma-Aldrich), 10 mg of hemin (Sigma-Aldrich), 1 mg of vitamin K (Sigma-Aldrich), 2 mL of bacitracin (50 mg mL^{-1}; Sigma-Aldrich), 1 mL of lincomycin (1 mg mL^{-1}; Sigma-Aldrich), 1 mL of nystatin (50 mg mL^{-1}; Sigma-Aldrich), 1 mL of bromocresol purple (2.5 mg mL^{-1}; Sigma-Aldrich), and 12 g of bacteriological agar (Oxoid). After incubation for 18 h at 37 °C in a 5% CO_2 atmosphere, the isolated colonies were picked from plates, purified by streaking on the same isolation medium, and stored at 4 °C as slant cultures until their characterization.

Bacterial isolate was identified by a polyphasic approach on the basis of its microscopical features (phase-contrast microscopy, shape, dimension, and presence of spores), biochemical characteristics (Gram stains, catalase activity), as well as by 16S rRNA gene sequencing. InstaGene^{TM} Matrix (Bio-Rad Laboratories, Hercules, CA, USA) was used to extract and purify the total genomic DNA according to the supplier's recommendations. Approximately 50 ng of DNA was used as template for PCR assay. Synthetic oligonucleotide primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') were used to amplify the 16S rRNA gene. The PCR mixture was prepared as described by Palomba *et al.* (2011), and PCR conditions were performed as described by Pepe *et al.* (2013). Amplicons, after visualization by agarose (1.5% w/v) gel electrophoresis at 100 V for 1 h, were purified using a QIAquick gel extraction kit (Qiagen S.PA, Milan, Italy) and sequenced. The DNA sequences were determined and analysed as previously reported (Pepe *et al.* 2011), and they were then compared to the GenBank nucleotide data library using the BLAST software (Altschul *et al.* 1990) on the informational website of the national centre of biotechnology (http://www.ncbi.nlm.nih.gov/Blast.cgi). The 16S rRNA gene sequence obtained from the bacterial strain *C. myxofaciens* BPM1 was submitted to the GenBank database, and the accession number is KT362364.

The nearly full-length 16S rRNA sequences of bacterial strain *C. myxofaciens* BPM1 and all strains belonging to the genera *Providencia*, *Morganella*, and *Proteus*, including *C. myxofaciens* (previously *Proteus myxofaciens*), were used to perform multiple nucleotide alignments using the ClustalW program (Thompson *et al.* 1994) from MEGA version 4.0 (Tamura *et al.* 2007). The nucleotide sequences of the strains were retrieved from the Ribosomal Database Project (RDP) (http://rdp.cme.msu.edu/) (Maidak *et al.* 2001). The phylogenetic tree was inferred using the neighbour-joining method with the Maximum Composite Likelihood model in MEGA4 program with bootstrap values based on 1,000 replications.

Chemical Production in Synthetic Substrate

C. myxofaciens BPM1 was pre-inoculated into 10 mL of brain heart infusion (BHI) broth (OXOID, Milan, Italy) and incubated for 18 h at 37 °C in a 5% CO_2 atmosphere (Novacell CO170 Incubator, VWR International PBI, Milan, Italy). The inoculum of *C. myxofaciens* BPM1 was performed by adding 1 mL (OD$_{550}$ 3.21; 2% v/v) of cultures from BHI broth into a flask containing 50 mL of MH medium (10 g L^{-1} polypeptone, 5 g L^{-1} yeast extract, 3 g L^{-1} K_2HPO_4, 2 g L^{-1} NaCl, 2 g L^{-1} $(NH_4)SO_4$, 0.2 g L^{-1} $CaCl_2$, 0.2 g L^{-1} $MgCl_2$, 10 g L^{-1} $MgCO_3$, 0.001 g L^{-1} Na_2S, pH 6.5) (Lee *et al.* 2002) supplemented with 20 g L^{-1} of glucose. The flasks were then incubated at 37 °C for 72 h in static conditions in a 5% CO_2 atmosphere (Novacell CO170 Incubator, VWR International PBI) or in anaerobic conditions (Whitley DG250 anaerobic workstation, Don Whitley Scientific, Shipley, England).

Samples were withdrawn periodically at 24-h intervals (0, 24, 48, and 72 h) and analysed for chemical production and glucose consumption by high-performance liquid chromatography (HPLC) (Refractive index detector 133; Gilson system; pump 307, column Metacarb 67 h (Varian) with flow 0.4 mL/min of H_2SO_4 0.01 N). All tests were performed in triplicate.

Chemical Production by Pretreated Lignocellulosic Biomass

Separate hydrolysis and fermentation (SHF) was performed to estimate the chemical production of *C. myxofaciens* BPM1 from pretreated lignocellulosic biomass (slurry at 20% water insoluble solids, WIS). Hydrolysis was performed by suspending 10 g of steam exploded pretreated lignocellulosic *Arundo donax* biomass in 50 mL of sodium acetate buffer (0.05 M, pH 5.0) obtaining a final concentration of WIS equal to 4%, and adding 69.63 filter paper unit (FPU) g^{-1} of solids of the commercial cellulase mixture Cellic® CTec2 (kindly provided by Novozymes, Bagsvaerd, Denmark). FPU of the enzyme Cellic® CTec2 was determined according to the standard procedure recommended by the International Union of Pure and Applied Chemistry (IUPAC) (Ghose 1987). Saccharification was conducted at 37 °C under shaking (120 rpm). Samples were withdrawn after 72 h of the saccharification process, and the glucose release was quantified by HPLC.

For SHF experiments, pretreated hydrolysed biomass (PHB) or the liquid fraction was used. The liquid fraction was either partially separated from solids by sedimentation under gravity and drawn using a peristaltic pump, or it was totally separated from solids and recovered by centrifugation (PHBc) at 5289 x g for 15 min. Fifty mL of PHB, PHBc, or PHBs were supplemented with MH components or with 20 g L^{-1} of corn steep liquor (CSL; Sigma-Aldrich, Milan, Italy) (Zheng *et al.* 2010) plus salt element solution (SE; 1.5 g K_2HPO_4, 1.5 g NaH_2PO_4, 0.2 $MgCl_2$, 0.2 $CaCl_2$, 10 g $MgCO_3$, and 1 mg Na_2S per liter) adjusting pH to 6.5.

All media were autoclaved at 120 °C for 15 min and then inoculated with *C. myxofaciens* BPM1 broth-culture (2% v/v) overnight. The SHF process was conducted in triplicate in static anaerobic conditions at 37 °C. Chemical production and glucose consumption were measured by HPLC periodically at 0, 24, 48, and 72 h.

Microbiological and Physico-Chemical Analyses

During the SHF process, the growth of *C. myxofaciens* BPM1 in the liquid cultures was determined by spreading 10-fold serial dilutions on the BHI solid medium. The plates were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere.

The pH was measured at the beginning of the SHF process and after 72 h using a pH meter (HI221, Hanna Instruments, Padova, Italy).

Statistical Analyses

One-way ANOVA followed by Duncan *post hoc* tests for pairwise comparison of means (at p ≤ 0.05) were used to assess the difference in the organic acids and ethanol production of *C. myxofaciens* BPM1 in different fermentation media. Statistical analyses were performed using SPSS 21.0 statistical software package (SAS Inc., Cary, NC) (Pallant 2013).

RESULTS AND DISCUSSION

Isolation and Identification of Bacterial Strain *C. myxofaciens* BPM1

      The selective cultural method used in this work allowed facultative anaerobic and/or microaerophilic bacteria to be isolated from rumen samples characterized by the presence of fermentative microorganisms, specifically potential organic acid-producing microbes. The two ionoporic antibiotics, lasalocid and monensin, were able to inhibit acetic acid-producing and hydrogen-producing microorganisms usually present in rumen, favouring the growth of succinic acid- and propionic acid-producing bacteria (Raja and Dhanasekar 2011). Among all isolates, the strain BPM1 was chosen for its unusual colony morphology. It is characterised at the phenotypic and molecular levels by a polyphasic approach. This strain was not spore-forming bacteria and had straight rod cells. It showed rhizoid, convex, and white colonies in SSIM. Finally, it was negative to a Gram reaction and positive to catalase assay.
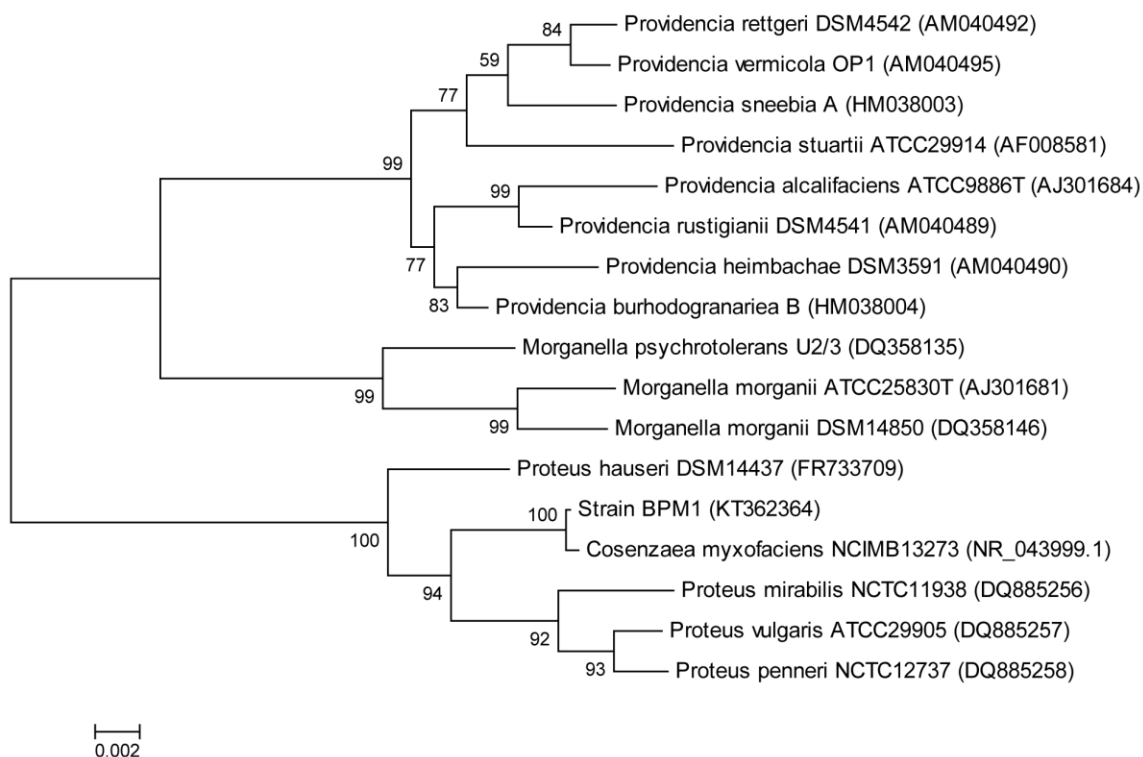
Fig. 1. Neighbour-joining tree based on the comparison of 16S rRNA gene sequences showing the relationships among strain BPM1 and 16 type strains belonging to the genera *Providencia, Morganella, Proteus,* and *Cosenzaea.* Bootstrap values (expressed as percentages of 1,000 replications) are given at the nodes. The sequence accession numbers used for the phylogenetic analysis are shown in parentheses following the species name. The scale bar estimates the number of substitutions per site.

      The BPM1 strain was also identified by sequencing the 16S rRNA gene. Using the BLAST software, the nearly full-length gene sequence (1,452 bp) of this bacterial strain showed 99% identity with *C. myxofaciens*, formerly known as *Proteus myxofaciens* and reclassified in the genus *Cosenzaea gen. nov.* as *Cosenzaea myxofaciens comb. nov.* (Giammanco *et al.* 2011). The genus *Cosenzaea* belongs to the family of Enterobacteriaceae as defined by Penner (2005) and Giammanco *et al.* (2011) and it

comprises only the species *C. myxofaciens* that was originally isolated from a larva of the gypsy moth (Cosenza and Podgwaite 1966). This work is reporting on the presence and isolation of this species from cattle rumen for the first time. In addition, *C. myxofaciens* is not a carrier of human disease (risk class 1), and it has no relevance in clinical studies (Toth and Emody 2000). Therefore, strains belonging to this species are more attractive for biotechnological applications because their manipulation does not require extensive safety precautions.

Moreover, to confirm molecular identification, a phylogenetic tree was constructed including 16 type strains belonging to the genera *Providencia*, *Morganella, Proteus*, and *Cosenzaea.* The results of neighbour-joining analysis of 16S rRNA sequences of the 17 strains are shown in the dendrogram depicted in Fig. 1. The closest relative species was *C. myxofaciens*, demonstrating that the strain BPM1 can be ascribed to this species.

Chemical Production in Synthetic Substrate

C. myxofaciens BPM1 was assayed using synthetic substrate (MH medium supplemented with glucose) in microaerophilic (5% CO_2, $T = 37\,°C$ for 72 h) and anaerobic conditions in order to determine its ability to produce bio-based chemicals for industrial applications as well as the best growth conditions to synthetize the highest amount of organic acids.

The *C. myxofaciens* BPM1 strain was able to produce a high amount of lactic acid anaerobically (Table 1). In fact, the highest lactate concentration recovered was 3.90 ± 0.09 g L^{-1} in microaerophilic and 8.93 ± 0.11 g L^{-1} in anaerobic conditions after 48 h and 24 h of fermentation, respectively (Table 1). However, the higher amount of lactic acid recovered in anaerobic than microaerophilic conditions could be explained by glucose consumption. In fact, this corresponded to 74% in anaerobic and to 26% in microaerophilic conditions after 24 h and 48 h of fermentation, respectively (Table 1).

During the fermentation process, the strain *C. myxofaciens* BPM1 was also able to produce organic acids such as succinate and acetate. In microaerophilic conditions, the highest amount of succinic and acetic acids (0.69 ± 0.01 g L^{-1} and 0.55 ± 0.02 g L^{-1}, respectively) was recovered after 72 h (Table 1). When *C. myxofaciens* BPM1 was incubated in an anaerobic environment, a significant increase of these organic acids was observed after 72 h of fermentation (with respect to microaerophilic conditions), reaching values of 1.11 ± 0.09 g L^{-1} and 1.57 ± 0.02 g L^{-1} for succinic and acetic acid, respectively (Table 1), although most of the fermentative products were synthetized in the first 24 h of growth (0.96 ± 0.13 g L^{-1} and 1.35 ± 0.04 g L^{-1}). These organic acids are known end products of anaerobic fermentative metabolism in some genera belonging to the family Enterobacteriaceae (Förster and Gescher 2014), and their synthesis is improved in the presence of a higher CO_2 concentration.

The simultaneous production of various fermentative metabolites from the same carbon source could determine the yield reduction of each acid. Metabolic engineering strategies and culture conditions could be developed to optimize the metabolic performance of the organic acid-producing species *C. myxofaciens* BPM1 and to reduce by-product formation (Song and Lee 2006). For example, several studies reported on the modification of metabolic pathway *via* overexpression of PEP or pyruvate-carboxylating enzymes and disruption of lactate dehydrogenase to improve succinic acid biosynthesis (Okino *et al.* 2008; Wang *et al.* 2011; Tsuji *et al.* 2013). Interestingly, the *C. myxofaciens* BPM1 produced a low amount of ethanol (approximately 1.5 g L^{-1}) in anaerobic conditions (Table 1).

Table 1. Concentrations (g L^{-1}) of Produced Metabolites and Consumed Glucose in MH Medium Supplemented with 20 g L^{-1} Glucose During Fermentation at 37 °C in Microaerophilic and Anaerobic Conditions*

TIME (h)	Microaerophilic Conditions[a]					Anaerobic Conditions[b]				
	Lactic Acid	Succinic Acid	Acetic Acid	Ethanol	Glucose	Lactic Acid	Succinic Acid	Acetic Acid	Ethanol	Glucose
0	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	20.00±1.12	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	20.00±1.20
24	3.49±0.12[B]	0.38±0.10[B]	0.00±0.00[A]	0.00±0.00[A]	17.43±1.05	8.93±0.11[E]	0.96±0.13[E]	1.35±0.04[C]	1.51±0.05[C]	5.25±0.56
48	3.90±0.09[C]	0.54±0.08[C]	0.52±0.03[B]	0.00±0.00[A]	14.79±0.95	8.45±0.22[D]	1.05±0.09[EF]	1.46±0.02[D]	1.64±0.06[D]	3.90±0.34
72	3.72±0.07[C]	0.69±0.01[D]	0.55±0.02[B]	0.00±0.00[A]	14.30±0.85	8.24±0.18[D]	1.11±0.09[F]	1.57±0.02[E]	1.32±0.06[B]	2.38±0.27

*Values represent the means ± SD of three replicates of independent experiments. Different letters indicate significant differences ($p \leq 0.05$).
[a] Microaerophilic conditions: a 5% CO_2 atmosphere
[b] Anaerobic conditions: atmosphere with 85% N_2, 10% CO_2, and 5% H_2

On the basis of the results obtained in these experiments, anaerobic conditions were chosen to investigate the ability of the strain *C. myxofaciens* BPM1 to synthetize organic acids from pretreated lignocellulosic biomass.

Fermentation Metabolite from Pretreated Lignocellulosic Biomass

The biotechnological performance of *C. myxofaciens* BPM1 was evaluated using pretreated biomass from *Arundo donax* in the separate hydrolysis and fermentation (SHF) process following various degrees of solids removal. The fermentations of pretreated *A. donax* were monitored over time using HPLC to measure fermentation metabolites (lactic acid, succinic acid, acetic acid, and ethanol) and glucose consumption. Although the production of bio-based chemicals, such as succinic acid, has been assayed and reported from a wide range of lignocellulosic materials such as corncobs, corn stover, rapeseed, rice, wheat straw, switchgrass, bagasse, and empty fruit bunches (Akhtar *et al.* 2014), the use of *A. donax* should be considered innovative. This lignocellulosic biomass was chosen for its low-cost production, high carbohydrate content, and ability to grow in marginal and contaminated lands (Fiorentino *et al.* 2013), making it an attractive substrate for bioconversion to biochemicals.

In SHF experiments using PHB as the sole carbon source without any additional nutrients, the strain *C. myxofaciens* BPM1 was not able to grow, demonstrated by the absence of glucose consumption (Fig. 2), nor was it able to synthetize organic acids (data not shown) due to a lack of nitrogen in the substrate (Lee *et al.* 2000). For this reason, nutritional components were added to the hydrolysed *A. donax* biomass in SHF experiments.

In these conditions, the strain *C. myxofaciens* BPM1 was able to produce lactic, succinic, and acetic acids as well as ethanol from the hydrolysis of *A. donax* biomass. Interestingly, the amount of organic metabolic compounds produced from hydrolysed lignocellulosic biomass was higher than that produced in the synthetic substrate for succinic (+51%), lactic (+36%), and acetic (+91%) acids, probably as a result of higher glucose consumption. As a consequence of acids production, the pH varied from 6.5 at the beginning of SHF experiments to 5.7-6.1 at the end of fermentation in all conditions.

Lactic acid was identified as the major end product, and its synthesis appeared to be influenced by the type of nutrient components added to the hydrolysed biomass. In fact, as shown in Table 2, lactate concentration was always significantly higher ($p \leq 0.05$) when CSL-SE was utilized, rather than MH, reaching maximum values after 72 h of fermentation in PHB+CSL-SE (12.13 ± 0.17 g L^{-1}) (Table 2). Moreover, these results highlighted that lactate production was not influenced by the presence of the solids fraction in the hydrolysed *A. donax* biomass. After 72 h of SHF, the concentration of lactate decreased from 10.65 to 9.87 and 8.42 g L^{-1} using PHB+MH, PHBs+MH, and PHBc+MH and from 12.13 to 11.84 and 11.15 g L^{-1} using PHB+CSL-SE, PHBs+CSL-SE, and PHBc+CSL-SE, respectively (Table 2). The production of lactic acid is a very interesting finding since it is a multifunctional organic acid used for a wide range of applications, such as in pharmaceutical, food, textile, and cosmetic industries, as well as in the production of biodegradable and biocompatible polylactic acid polymers (Taskila and Ojamo 2013). The chemical synthesis of lactic acid is an unsustainable technology at the industrial scale, but its synthesis by microbial fermentation is an eco-friendly and low-cost alternative to petroleum-derived products (Hu *et al.* 2015).

Table 2. Lactic Acid Concentration (g L^{-1}) During SHF Experiments in Anaerobic Conditions*

TIME (h)	PHB+MH[a]	PHB+CSL-SE[b]	PHBs+MH[c]	PHBs+CSL-SE[d]	PHBc+MH[e]	PHBc+CSL-SE[f]
0	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]
24	6.48±0.07[D]	7.53 0.08[E]	5.52±0.05[B]	8.42±0.12[G]	6.18±0.13[C]	6.25±0.05[C]
48	8.96±0.18[H]	8.95±0.11[H]	8.00±0.15[F]	10.59±0.09[K]	8.46±0.08[G]	10.31±0.24[J]
72	10.65±0.13[K]	12.13±0.17[N]	9.8 ±0.20[I]	11.84±0.08[M]	8.42±0.09[G]	11.15±0.20[L]

* The values represent the means ± SD of three replicates of independent experiments. Different letters after the values indicate significant differences (*p* ≤ 0.05).
[a] PHB+MH: hydrolysed biomass supplemented with MH components
[b] PHB+CSL-SE: hydrolysed biomass supplemented with Corn steep liquor (CSL) and salt element solution (SE)
[c] PHBs+MH: liquid fraction of hydrolysed biomass recovered after sedimentation and supplemented with MH components
[d] PHBs+CSL-SE: liquid fraction of hydrolysed biomass recovered after sedimentation and supplemented with Corn steep liquor (CSL) and salt element solution (SE)
[e] PHBc+MH: liquid fraction of hydrolysed biomass recovered after centrifugation and supplemented with MH components
[f] PHBc+CSL-SE: liquid fraction of hydrolysed biomass recovered after centrifugation and supplemented with Corn steep liquor (CSL) and salt element solution (SE)

Unlike lactic acid, the type of nutrient components added to the hydrolysed biomass did not influence succinate production, as shown by the statistical analysis. In fact, no significant differences in PHB, PHBs, and PHBc with CSL-SE or MH components were found (Table 3; *p* ≤ 0.05).

Table 3. Succinic Acid Concentration (g L^{-1}) During SHF Experiments in Anaerobic Conditions*

TIME (h)	PHB+MH[a]	PHB+CSL-SE[b]	PHBs+MH[c]	PHBs+CSL-SE[d]	PHBc+MH[e]	PHBc+CSL-SE[f]
0	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]
24	0.84±0.15[B]	0.93±0.05[B-D]	0.84±0.15[B]	0.93±0.05[B-D]	1.00±0.24[B-D]	0.86±0.24[BC]
48	1.08±0.17[B-G]	1.09±0.09[C-G]	1.04±0.21[B-E]	1.07±0.06[B-F]	1.25±0.31[E-H]	1.31±0.06[GH]
72	1.12±0.19[D-G]	1.28±0.19[F-H]	1.37±0.05[H]	1.41±0.05[HI]	1.60±0.03[IJ]	1.68±0.15[J]

* The values represent the means ± SD of three replicates of independent experiments. Different letters after the values indicate significant differences (*p* ≤ 0.05).
[a] hydrolysed biomass supplemented with MH components
[b] hydrolysed biomass supplemented with Corn steep liquor (CSL) and salt element solution (SE)
[c] liquid fraction of hydrolysed biomass recovered after sedimentation and supplemented with MH component
[d] liquid fraction of hydrolysed biomass recovered after sedimentation and supplemented with Corn steep liquor (CSL) and salt element solution (SE)
[e] liquid fraction of hydrolysed biomass recovered after centrifugation and supplemented with MH components
[f] liquid fraction of hydrolysed biomass recovered after centrifugation and supplemented with Corn steep liquor (CSL) and salt element solution (SE)

An inverse correlation between lactate and succinate production was observed, highlighting the fact that succinic acid production was influenced by the presence of the solids fraction in the hydrolysed biomass. In fact, *C. myxofaciens* BPM1 displayed

constantly increasing succinic acid concentration using PHB, PHBs, or PHBc as substrates under anaerobic conditions (Table 3). The concentration of succinate increased from 1.12 to 1.41 and 1.68 g L^{-1} using PHB, PHBs, and PHBc, respectively, showing that the centrifugation method was more effective at improving the fermentation activity of _C. myxofaciens_ BPM1. Using PHBc, in which the solids fraction was completely removed, a significant increase in succinic acid production was observed. In particular, after 72 h of SHF, 1.60 ± 0.03 and 1.68 ± 0.15 g L^{-1} of succinate was produced in PHBc+MH and PHBc+CSL-SE, respectively, in which 75 and 87% of glucose was consumed (Fig. 2). The presence or the partial removal of the solids fraction resulted in a lower concentration of succinic acid in PHB (24%) and PHBs (16%). However, a high cellular concentration of _C. myxofaciens_ BPM1 (9 and 8 Log CFU mL^{-1} after 48 and 72 h, respectively) was observed in all SHF experiments.
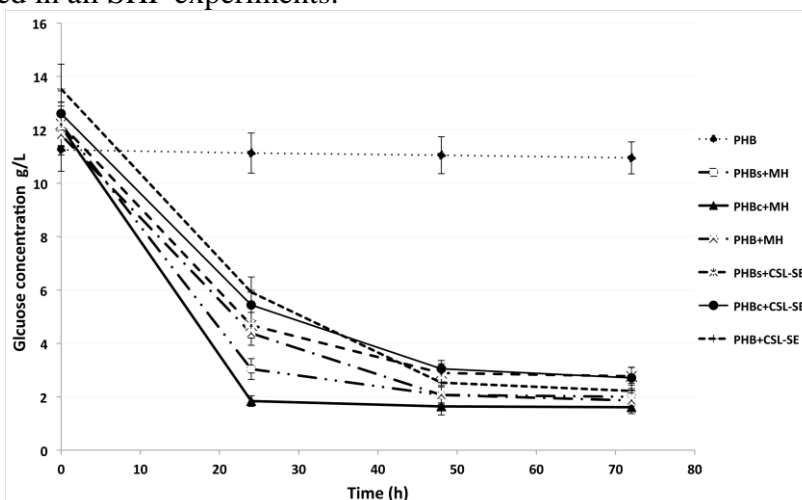
Fig. 2. Time courses of glucose concentrations during 72 h of fermentation in anaerobic conditions using hydrolysed _A. donax_ biomass

Table 4. Acetic Acid Concentration (g L^{-1}) during SHF Experiments in Anaerobic Conditions*

TIME (h)	PHB+MH[a]	PHB+CSL-SE[b]	PHBs+MH[c]	PHBs+CSL-SE[d]	PHBc+MH[e]	PHBc+CSL-SE[f]
0	3.01 ± 0.04^F	2.51 ± 0.05^{BC}	2.91 ± 0.06^E	2.44 ± 0.04^B	2.53 ± 0.02^C	2.33 ± 0.04^A
24	3.11 ± 0.08^G	2.49 ± 0.07^{BC}	3.92 ± 0.04^K	3.66 ± 0.04^I	2.98 ± 0.09^{EF}	2.66 ± 0.03^D
48	3.91 ± 0.04^K	3.57 ± 0.03^H	4.05 ± 0.05^M	3.79 ± 0.03^J	4.07 ± 0.05^M	3.88 ± 0.03^K
72	4.03 ± 0.02^{LM}	3.96 ± 0.02^{KL}	4.11 ± 0.05^M	3.92 ± 0.06^K	4.03 ± 0.07^{LM}	5.23 ± 0.04^N

* The values represent the means ± SD of three replicates of independent experiments. Different letters after the values indicate significant differences ($p \leq 0.05$).
[a] PHB+MH: hydrolysed biomass supplemented with MH components
[b] PHB+CSL-SE: hydrolysed biomass supplemented with Corn steep liquor (CSL) and salt element solution (SE)
[c] PHBs+MH: liquid fraction of hydrolysed biomass recovered after sedimentation and supplemented with MH components
[d] PHBs+CSL-SE: liquid fraction of hydrolysed biomass recovered after sedimentation and supplemented with Corn steep liquor (CSL) and salt element solution (SE)
[e] PHBc+MH: liquid fraction of hydrolysed biomass recovered after centrifugation and supplemented with MH components
[f] PHBc+CSL-SE: liquid fraction of hydrolysed biomass recovered after centrifugation and supplemented with Corn steep liquor (CSL) and salt element solution (SE)

Acetic acid production was not influenced by the concentration of solids when MH components were used as a nutrient supply. No significant differences were observed among PHB+MH, PHBs+MH, and PHBc+MH samples (4.03 ± 0.02, 4.11 ± 0.05, and 4.03 ± 0.07 g L^{-1}, respectively) after 72 h of SHF process (Table 4). On the contrary, with respect to PHB+CSL-SE and PHBs+CSL-SE, a significant increase was detected in PHBc+CSL-SE, reaching a plateau after 72 h of fermentation (5.23 ± 0.04 g L^{-1}). Additionally, acetic acid is a valuable product since it is an important feedstock for many chemicals (*e.g.*, calcium magnesium acetate, cellulose acetate, vinyl acetate polymer, acetic acid esters, acetic anhydride, *etc.*) that are usually made from petroleum-derived acetic acid (Balasubramanian *et al.* 2001). Therefore, the production of acetate from microbial fermentation of renewable source is a useful technology.

Finally, the strain was also able to produce relatively low amounts of ethanol from the hydrolysed biomass of *A. donax*. Similar to lactic acid, the production of ethanol seemed to be influenced by the type of the nutrient components added to the hydrolysed biomass. In particular, ethanol concentration was always significantly higher ($p \leq 0.05$) using MH components than CSL-SE as the nutrient supply, reaching maximum values of 1.39 ± 0.04, 1.03 ± 0.02, and 1.46 ± 0.03 g L^{-1} in PHB+MH, PHBs+MH, and PHBs+MH, respectively, after 72 h of fermentation (Table 5).

Table 5. Ethanol Concentration (g L^{-1}) During SHF Experiments in Anaerobic Conditions*

TIME (h)	PHB+MH[a]	PHB+CSL-SE[b]	PHBs+MH[c]	PHBs+CSL-SE[d]	PHBc+MH[e]	PHBc+CSL-SE[f]
0	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]	0.00±0.00[A]
24	0.90±0.07[D]	0.56±0.02[B]	0.56±0.04[B]	0.50±0.04[B]	1.18±0.02[F]	0.56±0.04[B]
48	1.03±0.05[E]	0.56±0.03[B]	0.91±0.06[D]	0.90±0.07[D]	1.23±0.07[F]	0.56±0.06[B]
72	1.39±0.04[G]	1.24±0.02[F]	1.03±0.02[E]	0.95±0.04[D]	1.46±0.03[H]	0.67±0.05[C]

*The values represent the means ± SD of three replicates of independent experiments. Different letters after the values indicate significant differences ($p \leq 0.05$).
[a] PHB+MH: hydrolysed biomass supplemented with MH components
[b] PHB+CSL-SE: hydrolysed biomass supplemented with Corn steep liquor (CSL) and salt element solution (SE)
[c] PHBs+MH: liquid fraction of hydrolysed biomass recovered after sedimentation and supplemented with MH components
[d] PHBs+CSL-SE: liquid fraction of hydrolysed biomass recovered after sedimentation and supplemented with Corn steep liquor (CSL) and salt element solution (SE)
[e] PHBc+MH: liquid fraction of hydrolysed biomass recovered after centrifugation and supplemented with MH components
[f] PHBc+CSL-SE: liquid fraction of hydrolysed biomass recovered after centrifugation and supplemented with Corn steep liquor (CSL) and salt element solution (SE)

Many studies have observed the simultaneous production of organic acids and ethanol in bacterial species such as *Mannheimia succiniciproducens*, *Bacillus* spp., *Clostridium* spp., and *Corynebacterium glutamicum* (Lee *et al.* 2006; Song *et al.* 2007; Rastogi *et al.* 2013; Yamauchi *et al.* 2014). However, the ability of *C. myxofaciens* species to synthesize lactate, succinate, acetate, and ethanol has not yet been reported.

The costs associated with the fermentation substrate, such as carbon and nitrogen sources, and the fermentation process are a limiting factor for the production of chemicals at an industrial scale. Therefore, the use of CSL as a nitrogen source is a low-cost

alternative to expensive components of the rich MH medium, such as yeast extract and peptone, that could also promote the production of undesired compounds as formate (Xi *et al.* 2013). Moreover, among the different processes, such as extraction, precipitation, and distillation, to separate and purify acids produced after fermentation, crystallization with a decreased pH is an inexpensive and effective environmentally friendly separation process (Huh *et al.* 2006).

CONCLUSIONS

1. A new bacterial strain that is able to synthetize several interesting chemicals was successfully isolated from bovine rumen using an ecological strategy.

2. The strain *C. myxofaciens* BPM1 efficiently fermented *A. donax* hydrolysate and produced lactate, succinate, and acetate. This is the first known report describing the occurrence of these activities in *C. myxofaciens* species.

3. The development of an inexpensive fermenting substrate using hydrolysed *A. donax* biomass and corn steep liquor as carbon and nitrogen sources could be suitable for economical and efficient production of biochemicals in industrial processes.

ACKNOWLEDGMENTS

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