(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 January 2019 (10.01.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/008131 A1

(51) International Patent Classification:
C12N 9/42 (2006.01) *C12P 7/62* (2006.01)
C12P 7/58 (2006.01) *C12N 1/22* (2006.01)

(21) International Application Number:
PCT/EP2018/068347

(22) International Filing Date:
06 July 2018 (06.07.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
P201730906 06 July 2017 (06.07.2017) ES

(71) Applicant: **CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS** [ES/ES]; C/ Serrano, 117, 28006 MADRID (ES).

(72) Inventors: **DVORAK, Pavel**; CENTRO NACIONAL DE BIOTECNOLOGIA (CNB), Calle Darwin, 3, 28049 Madrid (ES). **DE LORENZO PRIETO, Victor**; CENTRO NACIONAL DE BIOTECNOLOGIA (CNB), Calle Darwin, 3, 28049 Madrid (ES).

(74) Agent: **PONS ARIÑO, Ángel**; Glorieta Rubén Darío, 4, 28010 Madrid (ES).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2019/008131 A1

(54) Title: RECOMBINANT *PSEUDOMONAS PUTIDA* FOR THE PRODUCTION OF D-XYLONATE FROM D-XYLOSE

(57) Abstract: The invention refers to a recombinant *Pseudomonas putida* host cell capable of producing D-xylonate (D-xylonic acid) from D-xylose and a method for producing D-xylonate using the same. The recombinant *P. putida* host cell according to the present invention is, preferably, a *P. putida* EM42 strain which has been genetically modified to comprise and express a heterologous beta-glucosidase gene encoding a β-glucosidase enzyme from, preferably, the thermophilic Gram-positive bacterium *Thermobifida fusca*. This host cell of the invention is capable of growing in a minimal mineral culture medium comprising cellobiose as a sole carbon source. When applying the recombinant *P. putida* of the invention, it is possible to produce D-xylonate from D-xylose with high yield while using D-cellobiose as a sole carbon source for bacterial growth.

DESCRIPTION

Recombinant *Pseudomonas putida* for the production of D-xylonate from D-xylose

The invention belongs to the field of biotechnology, particularly to methods for the biotechnological production of value-added chemicals from lignocellulosic and cellulosic materials using improved recombinant microorganisms. Specifically, the invention relates to a recombinant Gram-negative bacterium which has been genetically modified to comprise a heterologous beta-glucosidase gene (*bglC* gene) from a thermophilic Gram-positive bacterium. This designed recombinant host cell may grow using cellobiose as a unique carbon source and produce D-xylonate from D-xylose with high yield. Therefore, this invention refers to this recombinant strain and a procedure for producing D-xylonate from D-xylose using the same.

STATE OF THE ART

Up to 220 million tonnes of lignocellulosic and cellulosic waste (such as crop residues, wood waste, paper, or food waste) are available for biotechnological purposes in the European Union every year. Lignocellulose can be decomposed to cellulose, hemicellulose, and lignin that can be further hydrolysed enzymatically to monomeric sugars (such as D-glucose or D-xylose) and lignin-derived aromatics, serving thus as cheap substrates for microbial fermentations and production of value-added chemicals (VAC).

D-glucose is a major product of enzymatic hydrolysis of cellulose when at least three different cellulolytic activities - endoglucanase, exoglucanase and β-glucosidase - are employed. Cellulases are usually supplied to the process in purified form and constitute most of the process cost. D-cellobiose, a disaccharide consisting of two D-glucose molecules linked by a β(1→4) bond, is the major product of cellulose hydrolysis when only two enzymes – endoglucanase and exoglucanase – are used.

D-xylose is a major monomeric component of hemicellulose. Beside other uses, D-xylose can serve as a substrate for biotechnological production of D-xylonic acid (D-xylonate).

D-xylonate was identified in 2004 by the US Department of Energy (DOE) as one of the 30 top lignocellulose-derived platform compounds that could be used for biotechnological production of VAC. D-xylonate was reported to be used as complexing agent or chelator, in dispersal of concrete, and as a precursor for compounds such as co-polyamides, polyesters, hydrogels, 1,2,4-butanetriol, ethylene glycol or glycolate. D-xylonate may also provide a cheap, non-food derived alternative for D-gluconic acid, which is widely used (about 80 kton/year) in pharmaceuticals, food products, solvents, adhesives, dyes, paints and polishes.

D-xylonate can be produced from D-xylose by microbial conversion. D-xylonate occurs naturally, being formed in the first step of oxidative metabolism of D-xylose by some archaea, bacteria, and fungi via the action of D-xylose or D-glucose dehydrogenases. Extracellular production of D-xylonate has been reported for several bacteria including *Gluconobacter oxydans* or *Pseudomonas fragi* (Buchert J, Puls J, Poutanen K. Appl. Microbiol. Biotechnol. 1988; 28:367–72). Several other microorganisms, including bacteria *Escherichia coli* or *Klebsiella pneumoniae* and yeasts *Saccharomyces cerevisiae* or *Kluyveromyces lactis*, were modified by genetic engineering towards D-xylonate production from D-xylose (Liu H, *et al.*, Bioresour. Technol. 2012; 115:244–8; Wang C, *et al.*, Appl. Microbiol. Biotechnol. 2016; 100:10055–63; Nygård Y, *et al.*, Metab. Eng. 2011; 13:383–91; Toivari M, *et al.*, Metab. Eng. 2012; 14:427–36). D-xylonate production using engineered *E. coli* was recently patented (US8637279). Furthermore, certain *Pseudomonads* were previously reported to be capable of D-xylose oxidation to D-xylonate (Toivari MH, *et al.*, Appl. Microbiol. Biotechnol. 2012; 96:1–8).

However, no commercial biotechnological production of D-xylonate has been established thus far. Major reasons of this are the low productivity of the mentioned recombinant microbial hosts, intracellular accumulation of product, undesired assimilation of the substrate, specially D-xylose, and D-xylonate for biomass formation which occurs, *e.g.,* in *Gluconobacter*, and the high cost of substrates (mostly D-glucose, D-galactose, or glycerol), co-substrates (*e.g.*, peptone, yeast extract or ethanol), and other expensive nutrients (*e.g.,* vitamins) that must be supplemented to the culture media of the reported microbial D-xylonate producers to achieve growth-associated formation of the target compound (Toivari MH, *et al.*, Appl. Microbiol. Biotechnol. 2012; 96:1–8). Growth of the host organism is needed in order to allow continuous regeneration of reducing co-factors, NAD(P)H or pyrroloquinoline quinone

(PQQ), required for functioning of cytoplasmic or membrane-bound dehydrogenases, respectively.

Hence, well defined microbial hosts capable of efficient substrate utilisation are highly desirable and they should ensure the production of the target compound with high yield and productivity while maintaining their continuous growth. These host cells could be used in simultaneous or separate saccharification and fermentation of cellulose for VAC production, and their application would reduce the cost and complexity of the process by omitting expensive supplements for their growth and purified cellulases from the culture medium.

In this sense, growth on cellobiose was previously established by expressing the *bglC* gene, encoding the *T. fusca* β-glucosidase, in the Gram-positive bacterium *Rhodococcus opacus* PD630 (Hetzler S, Steinbüchel A., Appl. Environ. Microbiol. 2013; 79:3122-5). Moreover, Lee J. and co-workers used *gh1-1* (NCU00130) β-glucosidase gene from *Neurospora crassa* in the industrially relevant *Corynebacterium glutamicum* ATCC 13032 (Lee J, *et al.*, Microb. Cell Factories. 2016; 15:20). It is also important to note that Tozakidis and co-workers (Tozakidis, IE, *et al.* Microbial cell factories. 2016; Vol.15: 103-115) expressed the *bglA* β-glucosidase gene from *Ruminiclostridium thermocellum* in *Pseudomonas putida*, but the enzyme was displayed on the surface of the host cell (*P. putida* strain KT2440) not produced intracellularly, and its activity, together with activity of other two displayed cellulases, was too low to allow the growth of the bacterium on the products of cellulose degradation. Tozakidis and co-workers also disclosed that only 20 µg/mL (i.e., 20 mg/L) of glucose was produced from the filter paper after 24 h by the action of three cellulases displayed on the surface of *P. putida* KT2440 (OD600 of cells in the reaction was 16.6) in the conditions reported in the article (Tozakidis, IE, *et al.* Microbial cell factories. 2016; Vol.15: 103-115). This amount of glucose is very low and not sufficient to support detectable growth of the cells. The authors themselves mention that β-glucosidases represent a known bottleneck in cellulose hydrolysis processes. To solve this, β-glucosidases either has to be substituted by an enzyme with higher activity, or the amount of β-glucosidase-displaying cells in the mixture has to be increased.

In this sense, display of the BglC enzyme on the surface allowed engineered *E. coli* to utilize cellobiose as a sole carbon source for growth and production of VACs such as isobutanol or ethanol (Desai SH, *et al.*, Appl. Microbiol. Biotechnol. 2014; 98:3727–36;

Muñoz-Gutiérrez I, *et al.*, Microb. Cell Factories. 2014; 13:106). Thus, Desai and co-workers (Desai, S.H., *et al.* Applied microbiology and biotechnology. 2014; Vol 98: 3727–3736) state that BglC β-glucosidase was either displayed on the surface of *E. coli* or excreted by the cells into the medium. The excretion system was found to be more efficient and allowed *E. coli* cells to grow on 20 g/L D-cellobiose with the specific growth rate μ of 0.20 h^{-1}.

In summary, well-defined microbial hosts capable of efficiently using a substrate as a sole carbon source, preferably a low-cost substrate, for their continuous growth while maintaining an efficient D-xylonate production from D-xylose are highly desirable in order to provide an industrially viable biotechnological production method of this compound. These microbial hosts should also have low nutrient demand for their growth, thus the profitability and simplicity of the D-xylonate production process would be improved.

DESCRIPTION OF THE INVENTION

The present invention provides a recombinant *Pseudomonas putida* host cell which expresses a heterologous beta-glucosidase enzyme, preferably the beta-glucosidase enzyme from *Thermobifida fusca* with SEQ ID NO: 1, and which is capable of efficiently utilizing D-cellobiose as a unique carbon source for growing while producing D-xylonate from D-xylose with high productivity. Thus, the invention also provides a method for producing D-xylonate from D-xylose using this recombinant host cell.

By applying this recombinant *P. putida* host cell of the invention it is possible to produce D-xylonate from D-xylose with high yield while reducing the production cost and complexity, since the strain uses D-cellobiose as a sole carbon source for bacterial growth and consequently no nutritional supplements need to be added to the culture medium.

The recombinant *P. putida* host cell expressing the beta-glucosidase enzyme from *Thermobifida fusca* with SEQ ID NO: 1, growing on D-cellobiose and producing D-xylonate from D-xylose according to the present invention, will be also referred to herein as "*P. putida* EM42beta" strain.

In the present invention, the recombinant *P. putida* EM42beta cell was designed by

introducing the heterologous *bglC* gene encoding the β-glucosidase (BglC) from the thermophilic cellulolytic Gram-positive bacterium *Thermobifida fusca* into the previously described *P. putida* EM42 strain (hereinafter "*P. putida* EM42 parental strain").

As shown in examples below, *P. putida* EM42 parental strain is capable of D-xylose oxidation to D-xylonate in a non-growing mode using a low D-xylose concentration in the presence of a minimal medium (**Fig. 1**). However, production of D-xylonate in this strain is not accompanied by the required cellular growth because the strain cannot use neither D-xylose nor D-xylonate for biomass formation. Thus, an additional carbon source needs to be supplemented to the culture medium in order to support the *P. putida* EM42 growth while promoting the conversion of D-xylose to D-xylonate. D-glucose was then added to the culture medium together with the D-xylose, nonetheless, as **Fig. 2** shows, glucose cannot be used for growth-associated D-xylonate production in this *P. putida* parental strain because the glucose dehydrogenase enzyme (Gcd) of the cell has higher affinity to D-glucose than to D-xylose. Therefore, when D-glucose was added as an additional carbon source to the culture medium, no D-xylose was converted to D-xylonate by the cells. Cellobiose could be used as an alternative carbon source instead of glucose, nevertheless *P. putida* EM42 parental strain is not able to grow on cellobiose. Consequently, this *P. putida* EM42 parental strain was engineered in the present invention towards growth on cellobiose giving rise to the recombinant host cell described in the present invention.

In the current invention, the advantages of the natural D-xylonate producer *P. putida*, that can efficiently form and release D-xylonate into the extracellular space, and the genetic engineering approach enabling the growth of the host cell in a minimal mineral medium with D-cellobiose supplemented as a sole carbon source, are combined. The present invention clearly shows that the beta-glucosidase enzyme from *T. fusca* is expressed intracellularly in *P. putida,* preferably in *P. putida* EM42beta*,* as it is show in **Fig. 4**. Moreover, the present invention shows that the beta-glucosidase enzyme from *T. fusca* expressed intracellarly in *P. putida,* preferably in *P. putida* EM42beta, allows the cells to grow on D- cellobiose (**Fig. 3**).

Therefore, the present invention describes a procedure for producing D-xylonate from D-xylose using the recombinant strain of the invention capable of growing on D-cellobiose.

Additionally, the recombinant strain described herein can serve as a suitable platform to be further engineered for the biotechnological production of D-xylonate-derived VACs such as ethylene glycol, glycolic or glyoxylic acid.

The recombinant *P. putida* host cell of the invention is the first reported Gram-negative bacterium for which efficient growth on cellobiose was achieved by virtue of intracellular expression and intracellular activity of a heterologous beta-glucosidase, preferably the beta-glucosidase from *T. fusca*. Furthermore, on contrary to the *E. coli* engineered cells previously described which were also capable of growing on cellobiose, the recombinant *P. putida* host cell of the invention presents the advantage that it has lower nutrient demand for growth whereas *E. coli* engineered cells growing on cellobiose have higher demands for growth medium such as B1 vitamin in the minimal culture medium. Thus, it is known that *E. coli* expressing a β-glucosidase in its surface grows on 20 g/L D-cellobiose with the specific growth rate μ of 0.20 h-1 (Desai SH, *et al.,* Appl. Microbiol. Biotechnol. 2014; 98:3727–36). In the present invention, it is shown that the recombinant cells, *P. putida* EM42beta which expressed the intracellular β-glucosidase from *T. fusca* grow on 5 g/L D-cellobiose as the unique carbon and energy source reached specific growth rate μ of 0.35 h^{-1}. Hence, the recombinant host cells, *P. putida*, of the present invention outperformed all known recombinant *E. coli* strains that has been reported to grow on D-cellobiose so far.

The recombinant strain of the invention is, therefore, capable of growing on cellobiose as a sole carbon source while producing D-xylonate from D-xylose with high yield and it does not require the supplementation of the culture medium with expensive substrates, co-substrates or additional nutrients, such as D-glucose, D-galactose, glycerol, peptone, yeast extract, vitamins, or the like. The recombinant strain of the invention has therefore low nutrient demand, allowing the reduction of costs and complexity of the industrial biotechnological methods for D-xylonate production.

Thus, a first aspect of the invention refers to a recombinant *Pseudomonas putida* host cell comprising a (heterologous and/or synthetic) nucleotide sequence encoding a heterologous β-glucosidase enzyme, wherein said heterologous β-glucosidase enzyme comprises an amino acid sequence that is at least 80%, preferably at least 81%, 82%, 83%, 84%, 85%, 86% 87%, 88% or 89%, more preferably at least 87%, even more preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to SEQ ID NO: 1. This host cell will be also referred to as “the host cell of the

invention", "the recombinant host cell of the invention", "the strain of the invention", "the cell of the invention", or the like.

"Pseudomonas putida" is a Gram-negative, rod-shaped, saprotrophic soil bacterium. It belongs to the phylum Proteobacteria, class Gammaproteobacteria, order Pseudomonadales, family Pseudomonadaceae, genus *Pseudomonas*, species *P. putida*.

The term "host cell" means a cell that is the receptor of an inserted foreign or heterologous sequence, preferably a nucleotide sequence. Thus, a recombinant host cell is a cell that comprises a heterologous and/or synthetic sequence, preferably a nucleotide sequence, inserted by genetic engineering techniques such that this cell comprising this sequence would not otherwise be found in nature. The "host cell" of the invention includes any cell or strain of *P. putida* species which is susceptible to transformation, transfection, transduction, and the like with a nucleic acid sequence, a nucleic acid construct or an expression vector comprising a polynucleotide encoding the heterologous beta-glucosidase enzyme as defined in this invention. In a preferred embodiment, the host cell of the invention is a *Pseudomonas putida* EM42 strain. Therefore, preferably, a *P. putida* EM42 strain (the "parental strain") has been modified in the present invention to comprise the nucleotide sequence encoding the heterologous beta-glucosidase enzyme as defined in this invention, wherein the expression of the beta-glucosidase enzyme is intracellular.

The *"Pseudomonas putida* EM42 strain" or "parental strain" is a derivative of the *P. putida* KT2440 strain with streamlined genome and improved physiological properties. The *P. putida* EM42 strain is described in, for instance but without limitation, Martínez-García E, *et al*., Microb. Cell Factories. 2014; 13:159.

The term "β-glucosidase" refers to an enzyme which catalyses the hydrolysis of a sugar dimer including, but not limited to, cellobiose, with the release of the corresponding sugar monomer/s. Beta-glucosidase enzyme acts upon β1->4 bonds linking two glucose or glucose-substituted molecules (*i.e.,* the disaccharide cellobiose). It has specificity for a variety of beta-D-glycoside substrates. It catalyzes the hydrolysis of terminal non-reducing residues in beta-D-glucosides with release of glucose.

The term "recombinant cell" means a cell in which a heterologous, foreign and/or

synthetic sequence, preferably a nucleotide sequence, more preferably a DNA or a cDNA sequence, even more preferably a cDNA sequence, has been inserted by means of genetic engineering techniques.

The term "heterologous", "foreign", "exogenous" or the like means a nucleotide sequence, for instance a gene or a gene coding sequence, or an amino acid sequence, for instance an enzyme or protein, that is artificially introduced or inserted into a cell which does not normally or naturally comprise or express said sequence. The term "heterologous" means therefore that the sequence derives from a different origin, organism or cell than the recipient organism or host cell.

Heterologous beta-glucosidases encompassed within the present invention are those endogenous (native, naturally expressed) beta-glucosidases derived from microorganisms such as bacteria other than *P. putida* or fungi, preferably bacteria, more preferably bacteria of the genus *Thermobifida.* Thus, in another preferred embodiment, the heterologous beta-glucosidase enzyme of the present invention is a beta-glucosidase from a bacterium belonging to the *Thermobifida* genus. In a more preferred embodiment, the bacterium belonging to the *Thermobifida* genus is *T. fusca*, *T. cellulosilytica* or *T. halotolerans*.

Examples of heterologous β-glucosidase enzymes that could be used in the present invention and which comprise an amino acid sequence that is at least 80% identical to SEQ ID NO: 1 are, but without limitation, the beta-glucosidase from *T. fusca* identified in the GenBank under the accession number WP_011291384.1, AAZ54975.1 or AAF37730.1 which are 100% identical to SEQ ID NO: 1, the beta-glucosidase from *T. cellulosilytica* identified in the GenBank under the accession number WP_068752922.1 which is 87% identical to SEQ ID NO: 1 or the beta-glucosidase from *T. halotolerans* identified in the GenBank under the accession number WP_084012700.1 which is 87% identical to SEQ ID NO: 1.

The term "identity" or "homology" as used herein, in the context of describing two or more polypeptide sequences, refers to a specified percentage of coincidences of amino acid residues at the positions from an alignment of two amino acid sequences. Methods of alignment of sequences for comparison are well-known in the art. The degree of identity can be determined by the Clustal method, the Wilbur-Lipman method, the GAG program, including GAP, BLAST or BLASTN, EMBOSS Needle and

FASTA. Furthermore, the Smith Waterman algorithm can be used in order to determine the degree of identity between two sequences. Preferably, in the present invention the degree of identity is determined by BLAST with the default parameters. More preferably, the degree of identity referred to in the present invention is at least 80%, more preferably at least 87%, even more preferably at least 90%, with regard to SEQ ID NO: 1 over a sequence length or query cover of at least 80%, more preferably at least 90%, even more preferably at least 99% or 100%.

In another preferred embodiment of the recombinant host cell of the invention, the β-glucosidase enzyme is a *Thermobifida fusca* β-glucosidase. Examples of *Thermobifida fusca* β-glucosidases are, without limitation, the β-glucosidases from *T. fusca* YX (GenBank accession numbers WP_011291384.1 or AAZ54975.1), *T. fusca* TM51 (GenBank accession number WP_011291384.1), *T. fusca* NBRC 14071 (GenBank accession number WP_011291384.1) or *T. fusca* ER1 (GenBank accession number AAF37730.1). In a more preferred embodiment, the β-glucosidase enzyme comprises, even more preferably consists of, the amino acid sequence SEQ ID NO: 1. When the host cell of the invention is the *Pseudomonas putida* EM42 strain and comprises a nucleotide sequence encoding the heterologous beta-glucosidase enzyme comprising the amino acid sequence SEQ ID NO: 1, this cell is also referred to in the present invention as "*P. putida* EM42beta strain".

The strain of the invention expresses intracellularly the heterologous β-glucosidase enzyme described herein. The term "expression" includes any step involved in the production of the heterologous beta-glucosidase enzyme by the host cell of the invention including, but not limited to, the transcription of the nucleotide sequence in an mRNA and the translation of said mRNA into a polypeptide. The host cell of the invention expresses intracellularly a functional heterologous beta-glucosidase enzyme as described herein. The term "functional" means that the expressed enzyme retains its beta-glucosidase activity, *i. e.* its capacity to hydrolyse sugar dimers, preferably cellobiose, acting upon β1->4 bonds.

The intracellular expression of the heterologous beta-glucosidase in the host cell of the invention may be performed by means of any method of genetic engineering known in the art, such as transformation, transfection, transduction or the like of a suitable *P. putida* host cell with a nucleic acid sequence encoding the heterologous beta-glucosidase, or a genetic construct comprising said nucleic acid sequence, and

cultivation of the modified host cell under conditions which induce the intracellular expression of said nucleic acid sequence.

Suitable nucleotide sequences encoding a heterologous beta-glucosidase enzyme as defined in the present invention are known in the art or can be designed based on the amino acid sequences given herein.

The nucleotide sequence encoding the heterologous beta-glucosidase can encode for the mature polypeptide or a preprotein consisting of a signal peptide linked to the mature enzyme which will have to be subsequently processed. Preferably, the nucleotide sequence encoding the heterologous beta-glucosidase encodes for the mature polypeptide. This nucleic acid sequence encoding the heterologous beta-glucosidase can be included in a gene construct, preferably in an expression vector, more preferably in a plasmid. Said genetic construct may further comprise one or more regulatory or control sequences of gene expression, such as promoters, enhancers, regulators, terminators, etc.

As shown in examples below, plasmid-based intracellular expression of the heterologous β-glucosidase enzyme resulted in fast growth of the host cell of the invention in a minimal culture medium with D-cellobiose as a sole carbon source (**Fig. 3**). Thus, the use of an extra-chromosomal plasmid and also the *bglC* gene expression from single copy on the chromosome of the host cell, allows intracellular and stable expression of the heterologous β-glucosidase enzyme in the host cell of the invention without the need for additional inducers. On the contrary, when the heterologous gene is included in the chromosome of the host cell, clones growing on cellobiose are also obtained (**Fig. 9**) but with slower D-cellobiose utilization compared to the plasmid-based system. Thus, in another preferred embodiment of the host cell of the invention, the nucleotide sequence encoding the heterologous β-glucosidase enzyme is comprised in a plasmid. In a more preferred embodiment, the plasmid comprises a constitutive promoter operably linked to the nucleotide sequence encoding the heterologous β-glucosidase enzyme.

In accordance with the present invention, a “nucleic acid sequence”, “polynucleotide” or “nucleotide sequence” is a nucleic acid molecule (polynucleotide) that can include DNA, RNA or derivatives of either DNA or RNA, including cDNA. Preferably, the nucleotide sequence referred to in the present invention is a cDNA coding sequence.

The nucleotide sequence encoding the heterologous beta-glucosidase enzyme disclosed in the present invention may be or not chemically or biochemically modified and may be artificially obtained by means of cloning and selection methods or by sequencing.

The term “gene construct” or "nucleic acid construct" as used herein relates to a functional unit required to transfer, and preferably express, a gene of interest, herein the nucleotide sequence encoding a heterologous beta-glucosidase enzyme as described, in a host cell. This term refers to a nucleic acid molecule, either single- or double-stranded, which is modified to contain segments of nucleic acids in a manner that would not otherwise exist in nature. The term “gene construct” is synonymous with the term "expression cassette" when the gene construct comprises the control sequences required for the expression of a coding sequence.

The term "expression vector" is defined herein as a linear or closed circular DNA molecule that comprises a polynucleotide encoding the heterologous beta-glucosidase enzyme as disclosed herein which is operably linked to additional nucleotides that provide for its expression. Said vector is introduced into the host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector.

The expression vector may be any vector (*e.g.*, a plasmid or virus, preferably a plasmid) which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleotide sequence of interest in the bacterial cell of the invention. The vector may be an autonomously replicating vector, *i.e.,* a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon may be used.

Examples of expression vectors that can be used in the present invention are any vector suitable for the expression of heterologous proteins in prokaryotes such as, but

without limitation, phages such as pSA3 or pAT28, cosmids, phagemids, yeast artificial chromosomes (YAC), bacterial artificial chromosomes (BAC), human artificial chromosomes (HAC), viral vectors such as lentiviral, retroviral or adenoassociated virus vectors or plasmids more preferably of the pET, pBR, pMB, pCR, RP, pUC, pPROK, pQE, pACYC, pBAD, pSC101, pMAL, pLEX, pSEVA, mp or pCold series, Bluescript or its derivatives, pKK233-2, YEP vectors, integration plasmids or centromere plasmids. Preferably, a plasmid of the pSEVA series is used in the present invention, more preferably pSEVA2213 or pSEVA238, even more preferably the low copy plasmid pSEVA2213 is used for the heterologous expression of the beta-glucosidase enzyme in the host cell according to the present invention.

In creating the expression vector, the coding nucleotide sequence is located in the vector so that it is operably linked to the appropriate control sequences for its expression. The expression vectors referred to in the present invention preferably comprise a polynucleotide encoding the heterologous beta-glucosidase enzyme described herein, a promoter and transcriptional and translational stop signals. The various nucleic acids and control sequences described herein may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleotide sequence encoding the enzyme at such sites.

The term "control sequences" is defined herein to include all components which are necessary or advantageous for the expression of a polynucleotide encoding the heterologous beta-glucosidase enzyme described in the present invention. Each control sequence may be native or foreign to the nucleotide sequence encoding the heterologous beta-glucosidase enzyme. Such control sequences may include, but are not limited to, a leader, an enhancer, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription and/or translation terminators. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating linkage of the control sequences with the coding region of the nucleotide sequence encoding the heterologous beta-glucosidase enzyme. The term "operably linked" denotes herein a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of the polynucleotide sequence such that the control sequence directs the expression of the coding sequence.

Furthermore, the vectors used in the present invention may comprise one or more selectable markers which permit easy detection and selection of the transformed, transfected, transduced, or the like cells. A “selectable marker” is a gene which expression product provides for a detectable signal, such as biocide or viral resistance, resistance to heavy metals, resistance to one or more antibiotics such as ampicillin, chloramphenicol, kanamycin, neomycin and/or tetracycline, prototrophy to auxotrophs, bioluminescence, chemoluminiscence, fluorescence such as luciferase, GFP, mCherry, and the like. Selectable markers for use in a bacterial host cell include, but are not limited to, *beta-lactamase, thymidine kinase, Neo, mutant FabI* or *URA3* genes, as well as equivalents or any combination thereof.

Vectors used in the present invention may contain an element(s) that permits integration of the vector into the host cell's genome (randomly or at specific sites) or autonomous replication of the vector in the cell independent of the genome. For integration into the host cell genome, the vector may rely on the polynucleotide's sequence encoding the heterologous beta-glucosidase enzyme or any other element of the vector for integration into the genome by homologous or non-homologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing integration by homologous recombination into the genome of the host cell at a precise location(s) in the chromosome(s).

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell. The origin of replication may be any plasmid replicator mediating autonomous replication which functions in a cell. The term "origin of replication" or "plasmid replicator" is defined herein as a nucleotide sequence that enables a plasmid or vector to replicate *in vivo*. Examples of origins of replication useful in a bacterial cell are pMB1, ColE1, p15A, *ori*S, *ori*V, R6K, RK2, pBBR1, RSF1010, pUC, pSC101, pBBR322/ROP, pRO1600/ColE1 and the like.
The promoter comprised in the plasmid may be, but without limitations, a constitutive or inducible promoter, although it is preferably a constitutive promoter. Examples of promoters that can be used in the present invention for the expression of the heterologous beta-glucosidase enzyme in the host cell of the invention are, without limitation, powerful promoters such as T7 or T5 promoter, trp promoter, lac promoter, tac promoter, PL promoter, pEM7 promoter, XylS/Pm promoter, cspA promoter and the like. In a particular embodiment, the promoter comprised in the plasmid according to

the present invention is the pEM7 constitutive promoter.

Examples of terminators that may be included in the plasmid of the invention are T7 terminator, fd phage terminator, T4 terminator, T500 terminator, T1 terminator, T0 terminator, tetracyclin resistant gene terminator, Escherichia coli trpA gene terminator, and the like.

In a particularly preferred embodiment, the plasmid referred to in the present invention for the expression of the heterologous beta-glucosidase enzyme in the host cell of the invention is the pSEVA2213 plasmid which comprises the pEM7 constitutive promoter operably linked to a nucleotide sequence encoding the heterologous beta-glucosidase enzyme as defined in the invention which preferably comprises or consists of SEQ ID NO: 1. This expression system allows the stable expression of the enzyme in the *P. putida* host cell without the need for additional expression inducer.

More than one copy of a polynucleotide encoding the heterologous beta-glucosidase enzyme described in the present invention may be inserted into the host cell to increase the production of the gene product. An increase in the copy number of the polynucleotide can be obtained by integrating at least one additional copy of the sequence into the host cell or by including an amplifiable selectable marker gene with the polynucleotide, where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the polynucleotide, can be selected by cultivating the cells in the presence of the appropriate selectable agent. The procedures used to link the elements described above to construct the recombinant expression vectors referred to in the present invention are well known to one skilled in the art.

The host cell of the invention can be cultivated in a nutrient medium suitable for the production of the heterologous beta-glucosidase enzyme using methods well known in the art. For example, the cell may be cultivated by shake flask cultivation, and small-scale or large-scale (including discontinuous, continuous, batch, fed-batch, solid state fermentations, or any combination thereof) in laboratory or industrial bioreactor in the presence of a suitable medium and under conditions allowing the heterologous beta-glucosidase enzyme to be expressed and/or isolated. The cultivation may take place in a suitable nutrient medium comprising, for instance, carbon and nitrogen sources and inorganic salts, using procedures known in the art. The culture need to have controlled

conditions of temperature, pH, agitation, proportion of gases (oxygen and carbon dioxide), etc., in addition to the presence of the adequate nutrients to permit cellular viability and division and the production of D-xylonate. This cell culture can be carried out in solid substrates, such as agar, or in a liquid medium which enables the cultivation of large amounts of cells in suspension.

The intracellularly expressed heterologous beta-glucosidase enzyme may be recovered using methods known in the art. For example, it may be recovered by conventional procedures including, but not limited to, cell lysing or cell disruption by means of mechanical and/or physical procedures, such as ultrasonic disrupting, French press disrupting or glass bead disrupting. In the case of lysing the cells, a method that uses lysozyme, peptidase, or a suitable combination thereof may be adopted. Subsequently, the enzyme may be recovered by, for instance, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

The heterologous beta-glucosidase enzyme produced in the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity of the protein for certain matrixes or chromatographic columns, hydrophobic, hydroxyapatite, chromatofocusing, size exclusion, based on molecular weight, purification tags, FPLC, HPLC), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction, in order to obtain a substantially pure beta-glucosidase enzyme that can be included in an enzymatic composition together with other cellulolytic enzymes.

The heterologous beta-glucosidase enzyme intracellularly expressed may be detected using methods known in the art that are specific for polypeptide detection. These detection methods may include, but without limitations, the use of specific antibodies anti- beta-glucosidase or an immunologically active peptide thereof, formation of an enzyme product (for instance, glucose), or disappearance of an enzyme substrate (for instance, cellobiose). Methods such as Western blot, ELISA or similar immunodetection techniques may be used for this analysis.

Another aspect of the invention refers to a composition comprising the recombinant host cell of the invention, hereinafter the “composition of the invention”. This composition may further comprise all those elements needed for the maintenance and

growth of the host cell of the invention in an *in vitro* culture and/or for the production of D-xylonate from D-xylose using the host cell of the invention such as, for instance but without limitation, Mg, Ca, Na, N, Cl, P, K, S, H, water, D-xylose, cellulosic and/or lignocellulosic biomass or hemicellulosic biomass or an hydrolysate of hemicellulosic material comprising D-xylose, a carbon source preferably comprising D-cellobiose, inorganic salts, bases and acids, solutions or buffers for controlling the pH preferably sodium phosphate buffer, and the like. More preferably, this composition of the invention further comprises salts and/or mineral nutrients suitable for the *in vitro* culture and growth of the host cell of the invention. Even more preferably, this composition of the invention comprises a M9 minimal culture medium which composition is well known in the art (Sambrook J, Maniatis T, Fritsch EF: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, N.Y.: Cold Spring Harbor Laboratory Press; 1989). The composition of the invention may further comprise any liquid, solid or semi-solid (gel) support for the host cell of the invention. Examples of these supports are, but without limitations, a matrix, a plate, a particle, preferably a nanoparticle, a gel, a biopolymer, beans, a liquid solution or suspension or the like.

The composition of the invention may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry composition. For instance, the composition may be in the form of a granulate or a microgranulate. The host cell of the invention included in this composition may be immobilized in a support as those described above in accordance with methods known in the art for the immobilization of bacterial cells.

The process known as “saccharification” is the degradation or hydrolysis of lignocellulosic and/or cellulosic biomass into fermentable sugars, *i. e.* the process of breaking complex carbohydrates (such as starch, lignocellulose or cellulose) into their monosaccharide components.

The host cell of the present invention may be used directly or as a source of the heterologous beta-glucosidase enzyme, and other cellulolytic enzymes, preferably in a sequential or simultaneous saccharification and fermentation process with lignocellulosic and/or cellulosic material, more preferably with hydrolysates of hemicellulosic material, even more preferably with D-xylose and D-cellobiose.

As evidenced by the examples below, the host cell of the invention may be used in the

production of D-xylonate from D-xylose in the presence of D-cellobiose as a sole carbon source in a minimal mineral culture medium. Thus, another aspect of the invention refers to the use of the recombinant host cell of the invention or the composition of the invention for the production of D-xylonate from D-xylose or from cellulosic or lignocellulosic biomass, preferably hemicellulosic biomass, or from a hydrolysate of hemicellulosic biomass wherein said hydrolysate comprises D-xylose. This use may be performed within a sequential or simultaneous saccharification and fermentation process with lignocellulosic and/or cellulosic material, more preferably with hydrolysates of hemicellulosic material comprising D-xylose and D-cellobiose, even more preferably with D-xylose and D-cellobiose.

The "D-xylonate" or "D-xylonic acid" or D-lyxonic acid" is a sugar acid that can be obtained by the complete oxidation of xylose. Its IUPAC name is (2*R*,3*S*,4*R*)-2,3,4,5-tetrahydroxypentanoic acid and its chemical formula is $C_5H_{10}O_6$.

The term "cellulosic or lignocellulosic biomass" means the biodegradable fraction of products, waste and residues from biological origin from agriculture (including plant, such as crop residues and animal substances), forestry (such as wood waste) and related industries including fisheries and aquaculture, as well as biodegradable fraction of industrial and municipal waste, such as municipal solid waste or wastepaper.

Another aspect of the invention refers to the use of the host cell of the invention or the composition of the invention as a platform to be further genetically engineered for the production of D-xylonate-derived value-added chemicals such as, for instance but without limitation, ethylene glycol, glycolic or glyoxylic acid, lactic acid, polyhydroxyalkanoates or derivatives of these compounds. This use or method refers to the modification of the cell in order to include engineered biochemical steps that, after a sequential or simultaneous saccharification and fermentation process in which the cell of the invention or the composition of the invention is cultured in a culture medium comprising D-xylose or cellulosic and/or lignocellulosic biomass or hemicellulosic biomass or an hydrolysate of hemicellulosic material comprising D-xylose, and an additional carbon source which is preferably D-cellobiose, lead to the production of value-added chemicals that can be recovered from the culture medium.

Another aspect of the invention relates to a method for producing D-xylonate from D-xylose, or from cellulosic or lignocellulosic biomass, preferably hemicellulosic biomass,

or from a hydrolysate of hemicellulosic biomass wherein said hydrolysate comprises D-xylose, hereinafter the "method of the invention", comprising the following steps:

a) culturing the host cell of the invention or the composition of the invention in a culture medium comprising D-xylose and an additional carbon source; and
b) recovering the D-xylonate produced from the culture medium after step (a).

In a preferred embodiment of the method of the invention, the culture medium of step (a) is a minimal mineral culture medium. A "minimal mineral culture medium" is a culture medium which comprises, but without limitation, water, Na_2HPO_4, KH_2PO_4, NaCl, NH_4Cl (or $(NH_4)_2SO_4$), $MgSO_4$ and $CaCl_2$, such as a M9 minimal culture medium. Alternatively, the minimal mineral culture medium may be a trace element solution of the composition described in Abril *et al.*, J Bacteriol. 1989 Dec; 171(12): 6782–6790, which comprises HBO_3, $ZnCl_2$, $MnCl_2$, $CoCl_2$, $CuCl_2$, $NiCl_2$, and $NaMoO_4$. More preferably, this culture medium of step (a) does not comprise nutrient supplementation with, for instance, co-substrates such as peptone, yeast extract or ethanol, nor with vitamins. Even more preferably, the culture medium of step (a) is M9 minimal culture medium or M63 minimal medium. M63 medium comprises KH_2PO_4, $(NH_4)_2SO_4$, $FeSO_4 \cdot 7H_2O$ and $MgSO_4$. In the most preferred embodiment, the culture medium of step (a) is M9 minimal culture medium.

In another preferred embodiment, the additional carbon source referred to in step (a) of the method of the invention comprises, preferably consists of, D-cellobiose. Thus, preferably, the culture medium does not comprise supplementation with another carbon source additionally to that mentioned in step (a) which is preferably D-cellobiose. Preferably, this culture medium does not comprise supplementation with glucose, galactose, glycerol or the like. In the most preferred embodiment, D-cellobiose is the sole carbon source comprised in the culture medium of the method of the invention for the growth of the host cell.

The term "supplementation" referred to a culture medium means the external addition of elements, preferably nutritional elements, to the medium.

In another preferred embodiment, the culture medium of step (a) further comprises cellulosic and/or lignocellulosic biomass, more preferably it comprises lignocellulose and/or hemicellulose, even more preferably it comprises hydrolysates of hemicellulose.

Preferably, D-cellobiose in step (a) of the method of the invention is in a constant concentration of between 2 and 50 g/L.

Preferably, D-xylose in step (a) of the method of the invention is in a constant concentration of between 2 and 100 g/L. Preferably, D-xylose concentration is 10 g/L.

Preferably, the amount of host cell in step (a) of the method of the invention is between 0.1 and 20 g/L dry cell weight. The host cell of the invention may be free or in any liquid, solid or semi-solid (gel) support. Examples of these supports are, but without limitations, a matrix, a plate, a particle, preferably a nanoparticle, a gel, a biopolymer, beans, a liquid solution or suspension or the like.

In another preferred embodiment of the method of the invention, the pH in step (a) is in a constant range between 5.5 and 8, preferably pH is between 6 and 7.5, more preferably pH is 7.0. Bases or acids (such as NaOH, KOH, HCl and/or H_2SO_4), or solutions or buffers comprising inorganic salts, preferably sodium phosphate buffer (NaP), may be added, continuously or when needed, to the culture medium in the method of the invention in order to control the pH.

In another preferred embodiment of the method of the invention, the temperature in step (a) is in a constant range between 15 and 40 °C, more preferably between 20 and 37 °C, even more preferably 30 °C.

In another preferred embodiment of the method of the invention, step (a) is performed during at least 12 hours, more preferably during 24h.

In another preferred embodiment of the method of the invention, step (a) is performed in agitation. The preferred range of RPM is between 100 and 1,000 rpm (depending on the shaker or bioreactor), higher agitation is more preferred.

The method of the invention may be integrated in a sequential or simultaneous saccharification and fermentation process.

A pretreatment process of the biomass is often required for increasing the access of the enzymes to their substrates and consequently to lead to an efficient hydrolysis.

Pretreatment uses various techniques including, but not limited to, ammonia fiber explosion, chemical treatment and steam explosion at high temperatures to alter the structure of cellulosic biomass and make cellulose more accessible. Thus, the method of the invention may further comprise a step, before step (a) or simultaneously with step (a), comprising the pretreatment of the biomass.

The method of the invention may be performed, for instance, in shake flask cultivation, and small-scale or large-scale (including discontinuous, continuous, batch, fed-batch, solid state fermentations, or any combination thereof) in laboratory or industrial bioreactor.

During the method of the invention the process conditions, such as volume of the culture medium, O_2, T^a, time, pH, the substrate (biomass as described above and/or D-xylose), carbon source for the cell growth (preferably D-cellobiose) or product concentrations (preferably D-xylonate) or the amount of host cell, may be monitored and controlled. Amounts of host cell of the invention, culture medium, substrate (D-xylose), carbon source (D-cellobiose), buffers for pH control, etc., may be added or removed, continuously or when needed, during the process of the invention in order to maintain the appropriate concentrations of each element and pH during the process.

Thus, preferably, the method of the invention takes place in the interior of an industrial bioreactor which comprises systems, probes and/or devices or, in general, any means suitable for monitoring and providing biomass of host cell of the invention, culture medium, substrate (D-xylose), carbon source (D-cellobiose), water, bases or acids for pH control, and the like. These suitable means are, for instance, valves and/or pipes connected from the bioreactor to one or more storage tanks. In the case of a host cell storage tank this is a culture tank in which an appropriate biomass of growing cells is maintained. The bioreactor wherein the method of the invention is taking place may further comprise systems or devices for controlling the reaction volume, O_2, pH, T^a and the like.

The bioreactor may further comprise one or more systems, preferably valves and pipes, for continuously or discontinuously recovering the D-xylonate produced.

The term “recovery” as used herein, refers to the collection of D-xylonate obtained after step (a) of the method of the invention. The recovery may be performed by any method

known in the art, including mechanical or manual ones. The recovery and purification can be performed, for instance, as described by Buchert J. *et al*. Biotechnol Lett (1986) 8: 541. doi:10.1007/BF01028079 with modifications described by Liu H. *et al*. Bioresour Technol. (2012) 115:244-8. doi: 10.1016/j.biortech.2011.08.065. Briefly, the cells are removed by centrifugation (preferably 2500 g for 10 min). The supernatant, which contains D-xylonic acid, is then decolorized in activated carbon. After activated carbon treatment, the supernatant is filtered and concentrated using rotary evaporator. The concentrate is again filtered in, preferably, 0.22 µm membrane and EtOH (3:1, v/v) is added to precipitate D-xylonic acid. The product is vacuum- dried, preferably for 12 h at 4 °C. The purified precipitate can be analysed by HPLC or NMR (^{13}C- and ^{1}H). Alternatively, the method described by Wang C. *et al*. Appl Microbiol Biotechnol. (2016) 100(23):10055-10063. DOI: 10.1007/s00253-016-7825-9 can be used. In this method, cell-free broth is passed through a column filled with anion exchange resin (D311), which had been treated with 1 mol/L NaOH. The column is rinsed with water to neutral pH and eluted with 1 mol/L NH_3 solution. The eluate is concentrated in a rotary evaporator and cooled for crystallization.

Another aspect of the invention relates to a process for the synthesis of polyhydroxyalkanoates (PHAs) comprising the following steps:

a. culturing the host cell of the invention or the composition of the invention in a culture medium comprising D-cellobiose; and
b. recovering the PHAs produced from the host cell or the composition of step (a).

Polyhydroxyalkanoates (PHA), commonly known as "bioplastics" are biodegradable polymers produced by certain bacteria, which accumulate inside the cell in the form of carbon source storage granules when environmental conditions are not optimal for growth. Therefore, the term bioplastic can be defined as a biopolymer synthesized from renewable sources, which can be biodegraded under controlled conditions and presents physicochemical characteristics similar to those of plastics derived from the Petrochemical industry. It is also important to note that PHAs may also be useful as biomaterials for biomedical applications due to their potential application in controlled drug delivery systems and tissue engineering.

In a preferred embodiment, the culture medium of step (a) is a minimal mineral culture medium as it is mentioned above. Specifically, all the conditions mentioned for the method of the present invention apply for the culture of the host cell of the invention for

the synthesis of PHAs.

In another preferred embodiment of the process for the synthesis of PHAs, it is characterized by the culture medium of step (a) comprises a limited nitrogen concentration. In a preferred embodiment, M63 medium or M9 minimal medium with reduced nitrogen has been used. In another preferred embodiment, the range of reduced nitrogen concentration the culture medium can be determined by the skilled person. In the present invention it is show that the limited nitrogen concentration range from 0.1 g/L to 0.5 g/L, preferably 0.2 g/L NH_4Cl in M9 medium or 0.2 g/L $(NH_4)_2SO_4$ in M63 medium.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skilled in the art to which this invention belongs. Methods and materials similar or equivalent to those described herein can be used in the practice of the present invention. Throughout the description and claims the word "comprise" and its variations are not intended to exclude other technical features, additives, components, or steps. Additional objects, advantages and features of the invention will become apparent to those skilled in the art upon examination of the description or may be learned by practice of the invention. The following examples and drawings are provided by way of illustration and are not intended to be limiting of the present invention.

DESCRIPTION OF THE DRAWINGS

FIG. 1. Conversion of D-xylose to D-xylonate using the pre-cultured cells of *Pseudomonas putida* EM42 (parental strain). **A)** Experiment conducted with cells of *P. putida* EM42 parental strain (EM42). **B)** Experiment conducted with cells of *P. putida* EM42 Δ*gcd* with deleted endogenous gene encoding glucose dehydrogenase (PP_1444). Cells were pre-grown in LB medium, washed with M9 minimal medium and resuspended in M9 medium with 5 g L^{-1} D-xylose to initial $OD_{600\ nm}$ of 0.55. Changes in $OD_{600\ nm}$ were followed by VIS spectrofotometry. Concentrations of D-xylose, D-xylonate, and D-xylono-γ-lactone in culture supernatants were determined using D-Xylose Assay Kit (Megazyme, USA) and hydroxamate method described by Lien (1959). Error bars represent standard deviations from two independent experiments.

FIG. 2. Test of conversion of D-xylose to D-xylonate in a culture of *Pseudomonas*

putida EM42 parental strain (EM42) growing on D-glucose. Cells were pre-cultured in LB medium, washed by M9 medium and resuspended in fresh M9 medium with 10 g L^{-1} D-glucose and 10 g L^{-1} D-xylose to initial $OD_{600\ nm}$ of 0.1. Cell growth was followed by measuring $OD_{600\ nm}$ using VIS spectrofotometry. Concentration of D-glucose in culture supernatants was measured using GO Glucose Assay Kit (Merck, USA). Concentrations of D-xylose, D-xylonate, and D-xylono-γ-lactone in culture supernatants were determined using D-Xylose Assay Kit (Megazyme, USA) and hydroxamate method described by Lien (1959). Error bars represent standard deviations from two independent experiments.

FIG. 3. Growth of *Pseudomonas putida* strains in M9 minimal medium with (**A**) 2.5 g L^{-1} D-cellobiose or (**B**) 5 g L^{-1} D-cellobiose used as a sole carbon source. "EM42beta" stands for the recombinant *P. putida* EM42 host cell of the invention bearing pSEVA2213_*bglC*. "EM42 SEVA2213" is a control strain bearing empty pSEVA2213 plasmid without *bglC* gene. Experiment was carried out in shaken flasks (30°C, 170 rpm). Growth data shown as mean ± SD from three independent experiments, sugar concentrations are shown as mean ± SD from two experiments.

FIG. 4. Detection of BglC using Western blot analysis. Enzyme with N-terminal 6xHis tag was detected (**1**) in cell-free extracts prepared from *Pseudomonas putida* EM42beta and **(2)** in a sample of culture supernatant using 6xHis mAb/HRP conjugate (Clontech, Laboratories, USA).

FIG. 5. Test of conversion of D-xylose to D-xylonate in a culture of *Pseudomonas putida* EM42beta growing on D-cellobiose. Cells were pre-cultured in LB medium, washed by M9 medium and resuspended in fresh M9 medium with 5 g L^{-1} D-cellobiose and 10 g L^{-1} D-xylose to initial $OD_{600\ nm}$ of 0.1. Cell growth was followed by measuring OD_{600nm} using VIS spectrofotometry. Concentration of D-cellobiose in culture supernatants was determined by HPLC. Concentrations of D-xylose, D-xylonate, and D-xylono-γ-lactone in culture supernatants were determined using D-Xylose Assay Kit (Megazyme, USA) and hydroxamate method described by Lien (1959). Error bars represent standard deviations from three experiments.

FIG. 6. Test of conversion of D-xylose to D-xylonate in a culture of *Pseudomonas putida* EM42beta growing in buffered M9 minimal medium with 5 g L^{-1} D-cellobiose and increased agitation of 275 RPM. **A)** Growth curves of EM42beta in M9 medium with 5 g

L^{-1} D-xylose and 50 mM sodium phosphate buffer (filled-in circles), 5 g L^{-1} D-xylose and 100 mM sodium phosphate buffer (open circles), 10 g L^{-1} D-xylose and 50 mM sodium phosphate buffer (filled-in diamonds), 10 g L^{-1} D-xylose and 100 mM sodium phosphate buffer (open diamonds). **B)** pH and D-xylonate detected in culture medium after 24 hrs of cultivation. Values in g L^{-1} and mM on X axis refer to concentrations of D-xylose and sodium phosphate buffer, respectively, in the culture medium.

FIG. 7. Overexpression of beta-glucosidases BglC, Ccel2454 and BglX in *Pseudomonas putida* EM42. The *bglC*, *ccel2454*, and *bglX* genes were separately cloned into pSEVA238 plasmids and plasmid constructs were transformed by electroporation into *P. putida* EM42 cells. **A)** Effect of induction of beta-glucosidase gene expression on growth of the host cells. Expression was induced at the time of 3 hrs by 3-methylbenzoate to the final concentration of 1 mM (time of induction indicated with black arrow). **B)** SDS-PAGE analysis of crude extracts obtained from the cultures described in part A). M, protein marker (molecular weights in kDa); 1, EM42 pSEVA238 empty vector; 2, EM42 pSEVA238_*bglC*; 3, EM42 pSEVA238_*ccel2454*; 4, EM42 pSEVA238_*bglX*. Bands of BglC, Ccel2454, and BglX of theoretical molecular weights of 54.2, 78.4, and 83.4 kDa are indicated with grey arrows. **C)** Comparison of specific activities of three beta-glucosidases in crude extracts. Activities were determined at 37°C in reaction mixture containing sodium phosphate buffer of pH 7.0 and 5 mM p-nitrophenol-beta-D-glucopyranoside used as a substrate.

FIG. 8. Growth of *Pseudomonas putida* KT2440 pSEVA2213_*bglC* in M9 minimal medium with 5 g L^{-1} D-cellobiose used as a sole carbon source. Experiment was carried out in shaken flasks (30°C, 170 rpm). Growth data shown as mean ± SD from three independent experiments, sugar concentrations are shown as mean ± SD from two experiments.

FIG. 9. Growth of *Pseudomonas putida* EM42_*bglC* C9 in M9 minimal medium with 5 g L^{-1} D-cellobiose used as a sole carbon source. Synthetic expression cassette with constitutive pEM7 promoter and *bglC* gene was introduced into the chromosome of EM42 and C9 candidate was selected based on its growth in M9 minimal medium with cellobiose. Experiments were carried out in shaken flasks (30°C, 170 rpm). Data shown as mean ± SD from two independent experiments.

FIG. 10. Growth of *E. coli* Dh5α pSEVA2213_*bglC* in M9 minimal medium with

thiamine HCl (1 mM) and 5 g L-1 D-cellobiose used as a sole carbon source. Experiments were carried out in shaken flasks (30°C, 170 rpm). Growth data shown as mean ± SD from three independent experiments, sugar concentrations are shown as mean ± SD from two experiments.

FIG. 11. Determination of polyhydroxyalkanoate (PHA) accumulation in *P. putida* EM42 recombinants by flow cytometry. Cell density and mean fluorescence of bacterial populations reached after 48 h of incubation in minimal medium with different carbon sources. *E. coli* Dh5α pSEVA2213 grown on 5 g L^{-1} D-glucose (*E. coli* GLU; negative control for PHA accumulation); *P. putida* EM42 pSEVA2213 grown on 30 g L^{-1} D-glucose (EM42 GLU; positive control for PHA accumulation); *P. putida* EM42 pSEVA2213_*bglC*_ grown on 15 g L^{-1} D-cellobiose (EM42 CEL); *P. putida* EM42 pSEVA2213 grown on 20 mM octanoate (EM42 OCT; positive control for PHA accumulation). Columns are mean ± standard deviation from at least three biological replicates.

EXAMPLES

The following examples are provided to illustrate the invention, but are not intended to limit the scope of the invention. Said examples are based on assays carried out by the inventors and show the high yield of D-xylonate production from D-xylose by the recombinant *Pseudomonas putida* EM42beta strain of the invention growing on D-cellobiose.

EXAMPLE 1. Production of D-xylonate by *P. putida* EM42 parental strain.

The aim of this assay was to test whether the parental strain, named *P. putida* EM42, can perform D-xylose oxidation to D-xylonate in non-growing mode using lower concentration of D-xylose in minimal medium with pre-cultured whole cells (**Fig. 1A**). Under defined conditions, 5 g L^{-1} of D-xylose was almost completely converted to D-xylonate which was detected in assay medium. D-xylono-γ-lactone was detected as intermediate of the conversion. No D-xylonate was detected in the control reaction employing EM42 strain with deleted *gcd* gene of glucose dehydrogenase (PP_1444) (**Fig. 1B**). These experiments proved that periplasmic membrane-bound Gcd, that plays crucial role also in peripheral glucose catabolic pathways, is responsible for D-xylose oxidation to D-xylono-γ-lactone in *Pseudomonas putida* strains KT2440 and

EM42. D-xylono-γ-lactone subsequently hydrolyses to D-xylonate spontaneously or the reaction can be catalysed by D-gluconolactonase (PP_1170).

However, this production of D-xylonate was not accompanied by cellular growth proving that the strain EM42 can utilize neither D-xylose nor D-xylonate for biomass formation. Thus, an alternative carbon source must be supplemented to the culture medium in order to support growth of *P. putida* and promote conversion of D-xylose to D-xylonate by PQQ co-factor regeneration.

D-glucose cannot be used for growth-associated D-xylonate production in *P. putida* because, as shown in a subsequent experiment, Gcd has higher affinity to D-glucose than to D-xylose. When D-glucose was added to the culture medium no D-xylose was converted to D-xylonate by EM42 cells (**Fig. 2**).

EXAMPLE 2. Recombinant expression of the heterologous beta-glucosidase enzyme from *Thermobifida fusca* in *P. putida* EM42 parental strain.

In view of the results obtained in Example 1 above, it was decided to engineer *P. putida* EM42 parental strain towards growth on an alternative carbon source, particularly on cellobiose. Intracellular cleavage of this disaccharide to two D-glucose molecules would allow engagement of Gcd for periplasmic oxidation of D-xylose while keeping the growth on cheap cellulose-derived substrate.

P. putida KT2440, and correspondingly also strain EM42, is not capable of growing on D-cellobiose though its genome contains *bglX* gene encoding a putative β-glucosidase (PP_1403) (**Fig. 3**, EM42 SEVA2213). Thus several β-glucosidases, including BglX, Ccel2454 (GenBank: ACL76783.1) from Gram-positive mesophilic cellulolytic bacterium *Clostridium cellulolyticum* and BglC (Tfu_0937, GenBank: AAZ54975.1, SEQ ID NO: 1) from Gram-positive thermophilic cellulolytic bacterium *Thermobifida fusca*, were screened for activity in *P. putida* EM42.

As shown in **Fig. 7A**, overexpression of *bglX* from pSEVA238 plasmid was toxic to the cells, thus this enzyme was disregarded. On the contrary, overexpression of *bglC* and *ccel2454* did not have a significant negative effect on cell viability when compared to the cells bearing empty pSEVA238 plasmid (EM42 pSEVA238, control). **Fig. 7C** shows that no beta-glucosidase activity was detected in crude extract with BglX and, despite

some activity was detected with Ccel2454, the highest beta-glucosidase activity was displayed with BglC. The selected heterologous enzyme to be expressed in *P. putida* EM42 strain was therefore BglC.

pSEVA2213 plasmid-based intracellular expression of the *bglC* gene resulted in fast growth of *P. putida* EM42 modified cell in minimal medium with D-cellobiose as a sole carbon source (**Fig. 3**, EM42beta). The strain expressing *bglC* gene from the low copy expression vector pSEVA2213 with the constitutive promoter pEM7 was designated "EM42beta". This selected expression system allows stable expression of BglC in *P. putida* cells without the need for additional expensive inducer. The *bglC* gene was alternatively introduced into the chromosome of *P. putida* EM42 (**Fig. 9**) and obtained clone designated *P. putida* EM42 C9 was shown to grow on cellobiose yet with somewhat slower D-cellobiose utilisation when compared to the plasmid-based system. *P. putida* EM42 with intracellular BglC growing on 5 g/L D-cellobiose as the unique carbon and energy source reached specific growth μ of 0.35 h^{-1}. Hence, to the best of our knowledge, the *P. putida* recombinant strain of the present invention outperformed all known recombinant *E. coli* strains that has been reported to grow on D-cellobiose so far.

Despite its origin, BglC activity was well compatible with optimal conditions for *P. putida* growth (30°C, pH 7.0). Interestingly, no heterologous cellobiose transporter had to be expressed in the *P. putida* recombinant cell to enable transport of disaccharide from culture medium to the cytoplasm. Western blotting with an antibody against 6xHis tag attached to the N-terminus end of the enzyme verified that BglC is produced intracellularly (**Fig. 4**). No enzyme was detected in culture supernatant (**Fig. 4**, lane 2). Similarly, activity of BglC was detected predominantly in cell-free extract (4.99 μmol min^{-1} mg^{-1} at 37°C, pH 7.0) while only traces of activity were detected in culture supernatant (3.68 μmol min^{-1} L^{-1}). No D-glucose, a product of D-cellobiose hydrolysis, was detected in culture supernatants by HPLC analysis (**Fig. 3**).

In a key proof-of-concept experiment, the EM42beta recombinant cells were grown in shaken flasks in 25 mL of minimal M9 medium with cellobiose of theoretical concentration of 5 g L^{-1} used as a sole carbon source and D-xylose of theoretical concentration of 10 g L^{-1} used as a substrate for production of D-xylonate (**Fig. 5**). The cells produced 5.88 g L^{-1} D-xylonate within 72 hrs of cultivation at 30°C. The yield was 0.66 g[g D-xylose]$^{-1}$ and volumetric productivity reached 82 mg L^{-1} h^{-1}. Volumetric

productivity during initial 24 hrs was 130 mg L^{-1} h^{-1}. The production of organic acid was accompanied by decrease of pH from 7.0 at time 0 hrs to 4.6 at the end of the cultivation. In follow-up experiment, M9 minimal medium with either 5 g L^{-1} or 10 g L^{-1} D-xylose was buffered with 50 mM or 100 mM sodium phosphate buffer to avoid possible inactivation of Gcd caused by rapid decrease of pH and cultivations were conducted using higher agitation (275 RPM) (**Fig. 6**, **Table 1**). Volumetric productivity during initial 24 hrs increased up to 2.6-times to 344 mg L^{-1} h^{-1} showing that the potential for further improvement of D-xylonate production in EM42beta recombinant cell under optimised culture conditions is high.

Table 1. D-xylonate and pH in culture medium after 24 hrs of cultivation of EM42beta recombinant cell in buffered M9 minimal medium with 5 g L^{-1} D-cellobiose and 5 or 10 g L^{-1} D-xylose.

Tested conditions	D-xylonate (g L^{-1})	Volumetric productivity (g L^{-1} h^{-1})	Final pH at the end of cultivation
5 g L^{-1} D-xylose, 50 mM NaP	4.14	172	6.27
5 g L^{-1} D-xylose, 100 mM NaP	4.69	195	6.37
10 g L^{-1} D-xylose, 50 mM NaP	8.25	344	5.97
10 g L^{-1} D-xylose, 100 mM NaP	8.02	334	6.08

abbreviations: NaP, sodium phosphate buffer

The inventors also show the growth of *P. putida* KT2440 pSEVA2213_*bglC* in M9 minimal medium with 5 g/L cellobiose. KT2440 is a parental strain of EM42 (**Fig. 8**). EM42 was prepared by genome streamlining of strain KT2440 as described previously. EM42 was shown (Martínez-García E, et al. Microb Cell Fact. 2014;13:159; Lieder S, et al. Microb Cell Fact. 2015;14:23) to have better physiological properties (e.g., higher expression of heterologous genes or higher energy charge) and is thus better suited for biotechnological tasks. The present results show that the strain KT2440, which bears pSEVA2213 plasmid with *bglC* gene, utilizes cellobiose slower (has longer lag phase) than strain EM42beta which bears the same plasmid and was also cultured in the same conditions. This data conclude that the recombinant strain EM42 is better than KT2440 strain for the purpose described herein.

Furthermore, in order to prove that the growth on cellobiose of the host cell of the invention can be achieved even when *bglC* gene is expressed from single copy on chromosome, the inventors introduced the *bglC* gene directly into the chromosome

(i.e., the strain contains no plasmid) in *P. putida* EM42_*bglC* obtaining the host cell *P. putida* EM42_*bglC* C9.

In order to obtain the *P. putida* EM42_*bglC* C9 the *bglC* gene with consensus RBS was subcloned into SacI and PstI sites of mini-Tn5-vector pBAMD1-4 (Martínez-García et al., Front. Bioeng. Biotechnol. 2014; 2, 462014a). Original pBAMD1-4 plasmid was endowed with pEM7 promoter subcloned into AvrII and EcoRI sites. *P. putida* EM42 cells (100 µL) were electroporated with plasmid DNA (200 ng) and recovered for 7 h in 5 mL of modified Terrific Broth (TB) medium (yeast extract 24 g L^{-1}, tryptone 20 g L^{-1}, KH_2PO_4 0.017 M, K_2HPO_4 0.072 M) at 30°C with shaking (170 rpm). Cells were collected by centrifugation (4000 rpm, 10 min) and resuspended in 100 mL of selection M9 medium with 5 g L^{-1} cellobiose and streptomycin (50 µg mL^{-1}). After four days of incubation at 30°C with shaking (170 rpm), cells were spun (4000 rpm, 15 min) and plated on selection M9 agar plates with 5 g L^{-1} cellobiose and streptomycin (50 µg mL^{-1}). Three fastest growing clones were re-streaked on fresh M9 agar plates with streptomycin or with streptomycin (50 µg mL^{-1}) and ampicillin (500 µg mL^{-1}) to rule out insertion of the whole pBAMD1-4 plasmid. The growth of three candidates in liquid minimal medium with cellobiose was verified. The insertion site of expression cassette (pEM7 promoter, bglC gene, T500 transcriptional terminator, and aadA gene) in chromosome of the fastest growing clone was determined by two-round arbitrary primed PCR with Arb6, Arb2, ME-O-Sm-Ext-F, and ME-O-Sm-Int-F described elsewhere (Martínez-García et al., Front. Bioeng. Biotechnol. 2014; 2, 462014a). Me-O-Sm-Int-F was used as a sequencing primer for PCR product. Position of bglC expression cassette in *P. putida* chromosome was reversely verified by colony PCR.

This data show that the growth on 5 g L^{-1} D-cellobiose can be achieved even when *bglC gene* is expressed from single copy on chromosome (**Fig. 9**). The advantage of this strain is that the *bglC* gene on chromosome is stably inherited without the need for recombinant plasmid in the cells. Though the cells bear antibiotic resistance gene now (aadA streptomycin/spectinomycin resistance; adenlytransferase) together with *bglC* gene for the purpose of easier selection/identification, the antibiotic resistance gene can be deleted from the chromosome. Recombinants lacking antibiotic resistance markers are more suitable for biotechnological purposes.

Additionally, the inventors show that *E. coli* cells transformed with the same plasmid which allowed *P. putida* EM42 (*E. coli* Dh5α pSEVA2213_*bglC*) to grow in minimal

medium with cellobiose were not able to grow on disaccharide in the same culture conditions as used for the cell of the invention (**Fig. 10**). This data state the advantage of using the *bglC* gene encoding the bglC protein (SEQ ID NO: 1) in the expression system in *P. putida* host disclosed in the present invention regarding other beta-glucosidases in other host cells (i.e. *E. coli*).

EXAMPLE 3. Production of polyhydroxyalkanoates (PHA) by the recombinant EM42beta cells of the invention growing on cellobiose.

P. putida KT2440 is well known for production of polyhydroxyalkanoates (PHA), biodegradable polyesters that have potential to substitute oil-derived plastics. Synthesis of biopolymer in *P. putida* was demonstrated from both fatty acids and unrelated substrates such as acetate, ethanol, glycerol, or some sugars, but never from cellodextrins such as D-cellobiose. Glucose can be converted to PHA via acetyl-CoA and de novo fatty acid biosynthesis. Production of PHA in *P. putida* is promoted under nitrogen limitation (Prieto, A., *et al.* Environ Microbiol. 2016; 18:341-57).

To qualitatively determine formation of PHA by EM42beta recombinant cell of the invention the procedure disclose by Spiekermann, P. *et al* (Spiekermann, P., et al. Arch Microbiol. 1999; 171:73-80) was followed.

Cells, particularly, *E. coli* Dh5α pSEVA2213 (negative control which does not produce PHA), *P. putida* EM42 pSEVA2213 (grown on glucose or octanoate -positive controls for PHA formation), and *P. putida* EM42beta (grown on cellobiose to test formation of PHA on this substrate) were pre-grown overnight in LB medium with appropriate antibiotic, collected by centrifugation and washed by 0.1 N M63 minimal medium with limited nitrogen content (per 1 L: 13.6 g KH_2PO_4, 0.2 g $(NH_4)_2SO_4$, 0.5 mg $FeSO_4$ $7H_2O$, pH adjusted to 7.0 with KOH). M63 is a minimal, low osmolarity media, resulting in slower growth rate of the cells. Also M9 minimal medium with reduced nitrogen content (per 1 L: 4.25 g Na_2HPO_4 $2H_2O$, 1.5 g KH_2PO_4, 0.25 g NaCl, 0.2 g NH_4Cl) can be used. Cells were resuspended to OD600 of 0.1 in 10 mL of 0.1 N M63 medium, added with 2 mM MgSO4, 2.5 mL L^{-1} trace element solution (Abril, M.A., *et al.* J. Bacteriol. 1989; 171, 6782–6790.), appropriate antibiotic and carbon source (30 g L^{-1} glucose or 15 g L^{-1} cellobiose). *E. coli* Dh5α pSEVA2213 grown in M63 medium with additional thiamine HCl (5 mg L^{-1}) and 5 g L^{-1} glucose and *P. putida* EM42 pSEVA2213 grown in M63 medium with 20 mM octanoic acid was used as a negative and positive

control, respectively, for PHA accumulation. Cells were cultured for 48 h at 30 °C with shaking (170 rpm). PHA positive cells were determined with flow cytometry assay following the protocol of Tyo and co-workers (Tyo, K.E., *et al.* Appl Environ Microbiol. 2006; 72:3412-7) with minor modifications. Briefly, cell cultures were cooled down in an ice bath for 15 min. Cells were collected by centrifugation (5,000 g, 4 °C, 5 min), pellets were resuspended to A_{600} of 0.4 in cold TES buffer of pH 7.5 (10 mM Tris-HCl, 10 % (w/v) sucrose, 2.5 mM EDTA), and incubated on ice for another 15 min. Cells were spun again and resuspended in the same volume of ice cold 1 mM $MgCl_2$. Nile Red solution of 1 mg mL^{-1} concentration was added (3 µL) to an aliquot (1 mL) of the cell suspension, which was then incubated for 30 min in the dark at 4 °C. Fluorescence of cells was analysed using MACSQuant VYB cytometer (Miltenyi Biotec, Germany). For excitation an Ar laser (543 nm, diode-pumped solid state) was used and the fluorescence of Nile red was detected at 598 nm using a 614/50 nm band-pass filter. At least 50,000 cells were analyzed in each sample. FlowJo v.10 software (FlowJo, USA) was used for data processing.

The data show that 48±0.03 % of cells cultured on cellobiose was PHA positive. PHA positive fraction of *P. putida* pSEVA2213 cells grown on 30 g L^{-1} glucose was 61±0.06 %. The instant example proved that recombinant *P. putida* EM42 metabolizes cellobiose, accordingly to monomeric glucose, to acetyl-CoA and can utilize this new substrate for production of desired biopolymer.

Therefore, the data of the present invention show that:

1. Both *P. putida* KT2440 and its derivative EM42 can use D-cellobiose as a sole carbon and energy source and can grow on this disaccharide.
2. *P. putida* EM42 can grow on D-cellobiose when *bglC* gene is on plasmid or stably introduces in the chromosome.
3. *E. coli* with beta-glucosidase on pSEVA2213 plasmid cannot grow on D-cellobiose under the same conditions which were used for *P. putida* (note that M9 medium for *E. coli* contains also thiamin HCl that is required by Dh5α strain), despite the fact that reasonably high BglC activity (1.51±0.12) was detected in the cell-free extracts prepared from *E. coli* cells expressing *bglC* gene.
4. *P. putida* metabolizes D-cellobiose similarly as D-glucose through acetyl-CoA which can serve as a precursor for production of PHA, a bacterial biodegradable polymer that has potential to substitute oil-derived plastics.

The project leading to this application has received funding from the European Union's Horizon 2020 research and innovation programme under the Marie Sklodowska-Curie grant agreement No 704410.

CLAIMS

1. A recombinant *Pseudomonas putida* host cell comprising a nucleotide sequence encoding a heterologous β-glucosidase enzyme, wherein said heterologous β-glucosidase enzyme comprises an amino acid sequence that is at least 80%, preferably at least 87%, more preferably at least 90%, identical to SEQ ID NO: 1, and wherein said β-glucosidase enzyme is expressed intracellularly.

2. The recombinant host cell according to claim 1, wherein the β-glucosidase enzyme is a *Thermobifida fusca* β-glucosidase.

3. The recombinant host cell according to any of claims 1 or 2, wherein the β-glucosidase enzyme comprises the amino acid sequence SEQ ID NO: 1.

4. The recombinant host cell according to any one of claims 1 to 3, wherein said host cell is a *Pseudomonas putida* EM42 strain.

5. The recombinant host cell according to any one of claims 1 to 4, wherein the nucleotide sequence encoding the heterologous β-glucosidase enzyme is comprised in a plasmid.

6. The recombinant host cell according to claim 5, wherein the plasmid comprises a constitutive promoter operably linked to the nucleotide sequence encoding the heterologous β-glucosidase enzyme.

7. A composition comprising the recombinant host cell according to any one of claims 1 to 6.

8. Use of the recombinant host cell according to any one of claims 1 to 6 or the composition according to claim 7 for the production of D-xylonate from D-xylose.

9. A method for producing D-xylonate from D-xylose comprising the following steps:

a) culturing the host cell according to any one of claims 1 to 6 or the composition according to claim 7 in a culture medium comprising D-xylose and an additional carbon source; and

b) recovering the D-xylonate produced from the culture medium after step (a).

10. The method according to claim 9, wherein the additional carbon source comprises, preferably consists of, D-cellobiose.

11. The method according to any of claims 9 or 10, wherein the pH in step (a) is in a range between 5.5 and 8, preferably 7.0.

12. The method according to any one of claims 9 to 11, wherein the temperature in step (a) is in a range between 15 and 40 °C, preferably 30 °C.

13. The method according to any one of claims 9 to 12, wherein the step (a) is performed during at least 12 hours, more preferably during 24h.

14. The method according to any one of claims 9 to 13, wherein the step (a) is performed in agitation.

15. The method according to any one of claims 9 to 14, wherein the culture medium comprises lignocellulose and/or hemicellulose.

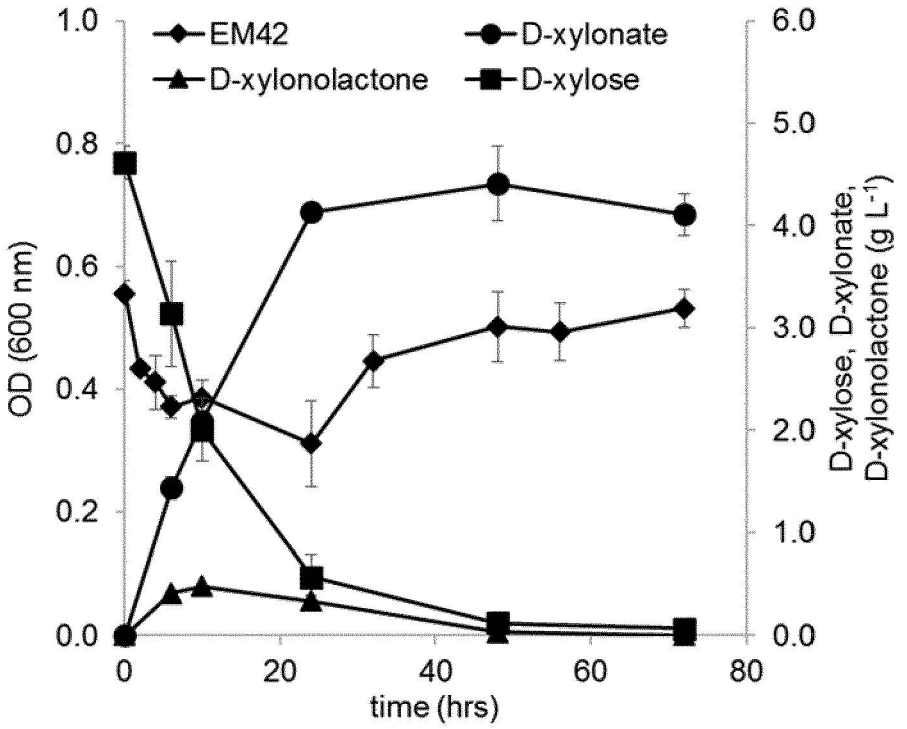
16. A process for the synthesis of polyhydroxyalkanoates (PHAs) comprising the following steps:

a. culturing the host cell according to any one of claims 1 to 6 or the composition according to claim 7, in a culture medium comprising D-cellobiose; and
b. recovering the PHAs produced from the host cell or the composition of step (a).

17. The process according to claim 16 wherein the culture medium of step (a) comprises a limited nitrogen concentration, preferably in the range of 0.1 g/L to 0.5 g/L.

FIG. 1

A


EM42
D-xylonate
D-xylonolactone
D-xylose
OD (600 nm)
D-xylose, D-xylonate, D-xylonolactone (g L-1)
time (hrs)
1.0
0.8
0.6
0.4
0.2
0.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
20
40
60
80

B

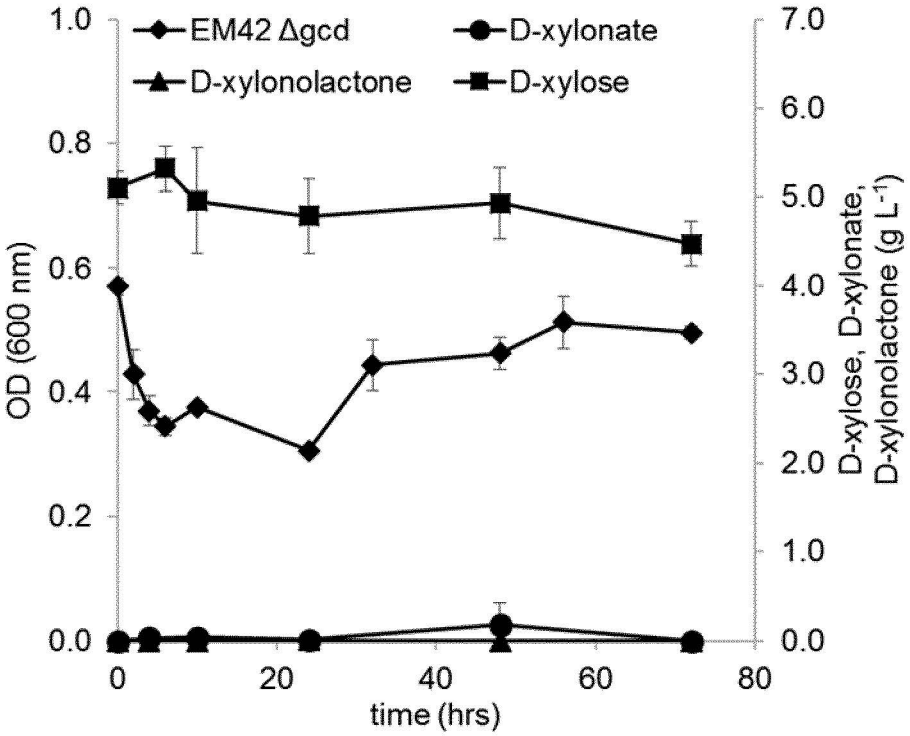

EM42 Δgcd
D-xylonate
D-xylonolactone
D-xylose
OD (600 nm)
D-xylose, D-xylonate, D-xylonolactone (g L-1)
time (hrs)
1.0
0.8
0.6
0.4
0.2
0.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
20
40
60
80

FIG. 2

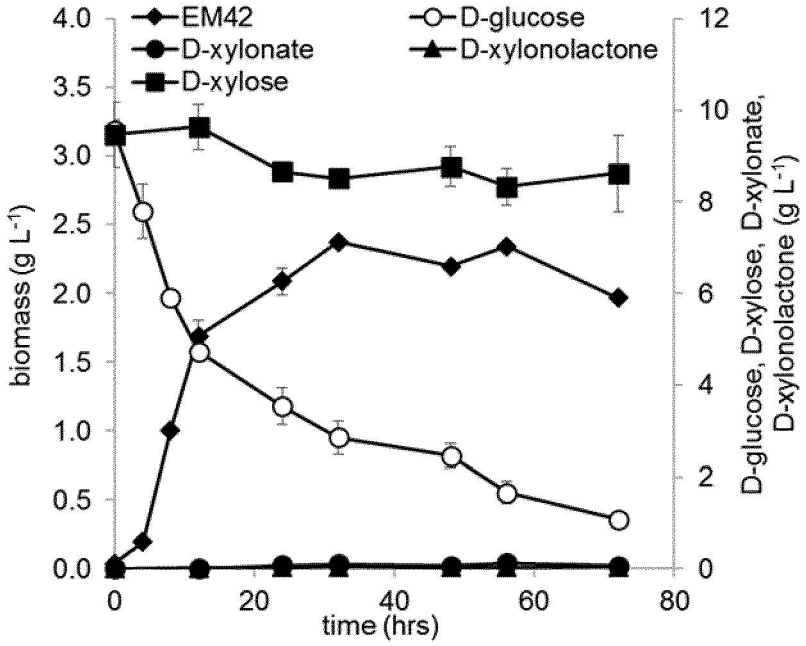

EM42
D-glucose
D-xylonate
D-xylonolactone
D-xylose
biomass (g L-1)
D-glucose, D-xylose, D-xylonate, D-xylonolactone (g L-1)
time (hrs)

FIG. 3

A

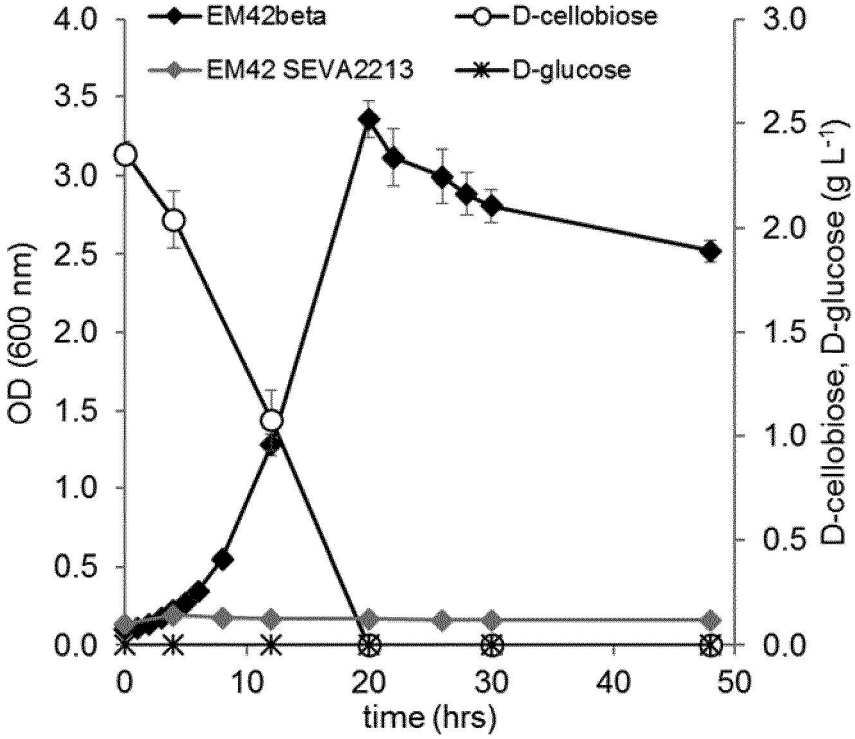

EM42beta
D-cellobiose
EM42 SEVA2213
D-glucose
OD (600 nm)
D-cellobiose, D-glucose (g L-1)
time (hrs)

FIG. 3 (cont.)

B

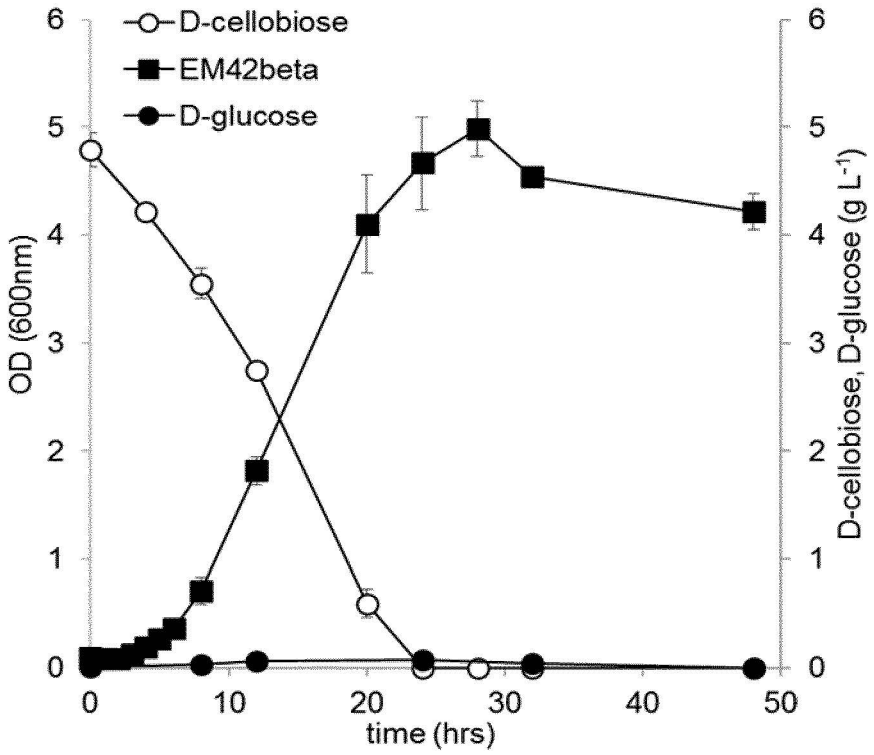


FIG. 4

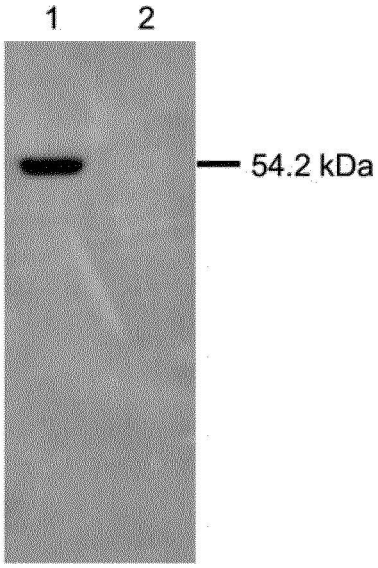


FIG. 5

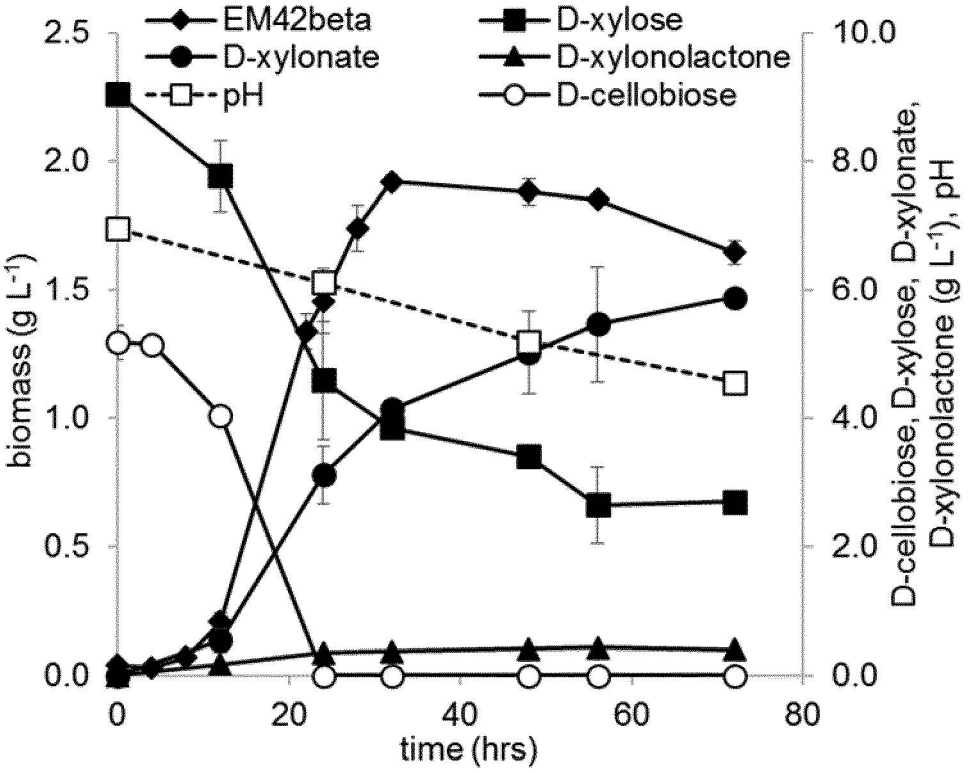
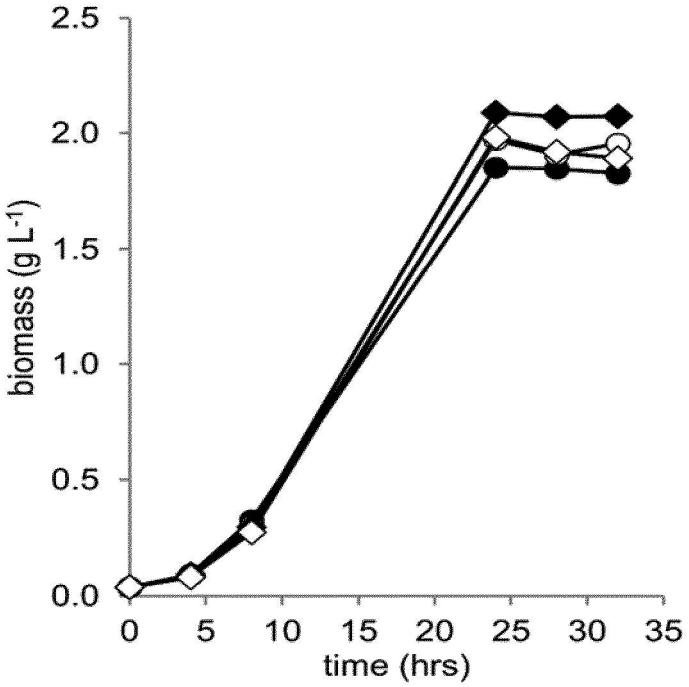

EM42beta
D-xylose
D-xylonate
D-xylonolactone
pH
D-cellobiose
biomass (g L-1)
D-cellobiose, D-xylose, D-xylonate, D-xylonolactone (g L-1), pH
time (hrs)
0.0
0.5
1.0
1.5
2.0
2.5
0
20
40
60
80
0.0
2.0
4.0
6.0
8.0
10.0

FIG. 6

A


2.5
2.0
1.5
1.0
0.5
0.0
biomass (g L-1)
0
5
10
15
20
25
30
35
time (hrs)

B

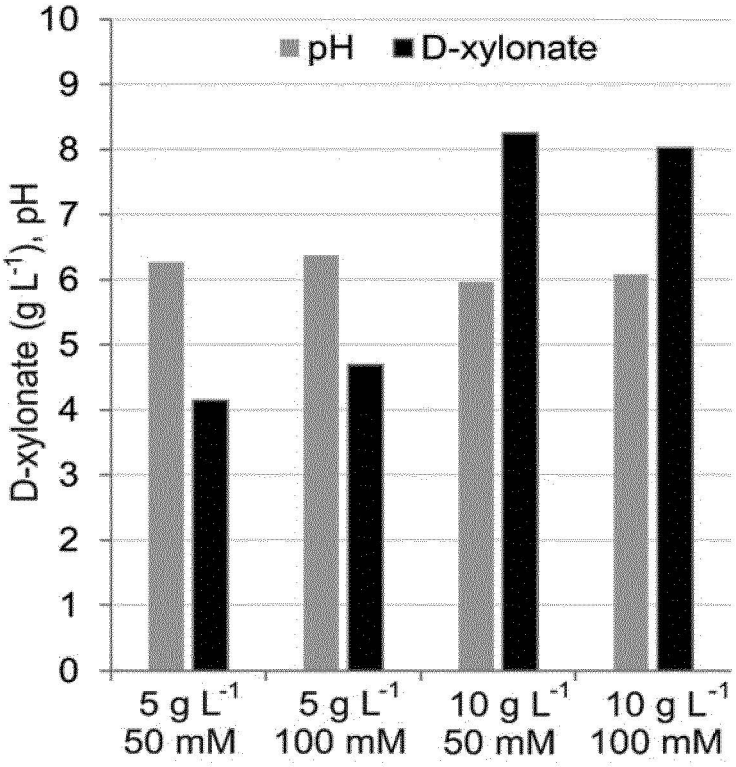
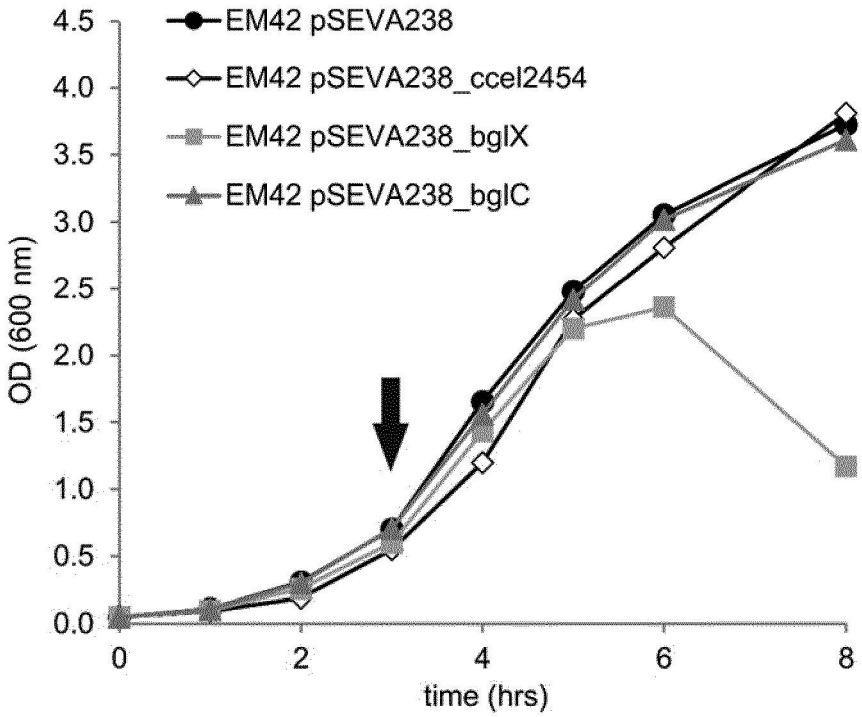

pH
D-xylonate
10
9
8
7
6
5
4
3
2
1
0
D-xylonate (g L-1), pH
5 g L-1 50 mM
5 g L-1 100 mM
10 g L-1 50 mM
10 g L-1 100 mM

FIG. 7

A


EM42 pSEVA238
EM42 pSEVA238_ccel2454
EM42 pSEVA238_bglX
EM42 pSEVA238_bglC
OD (600 nm)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
time (hrs)

B

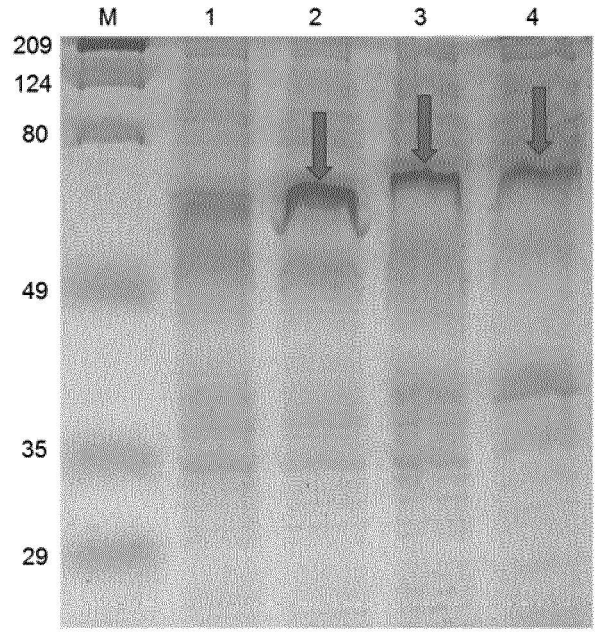

M
1
2
3
4
209
124
80
49
35
29

FIG. 7 (cont.)

C

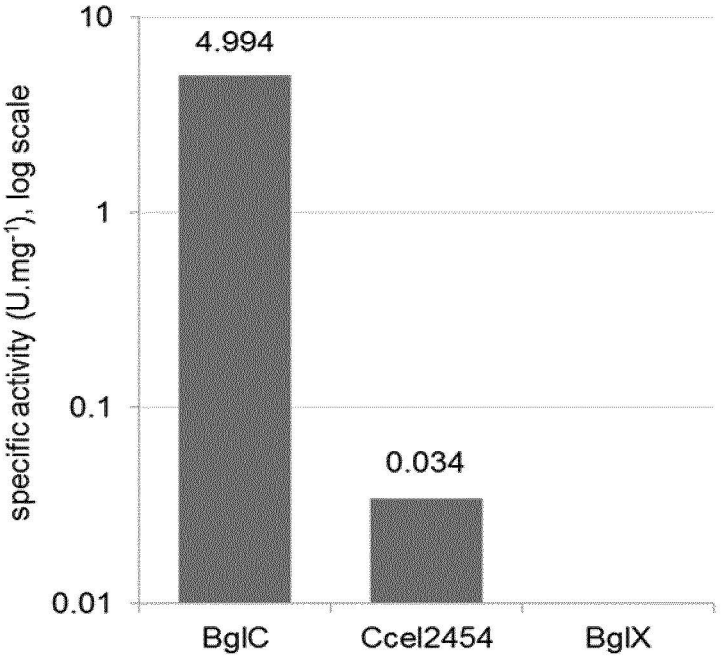


FIG. 8

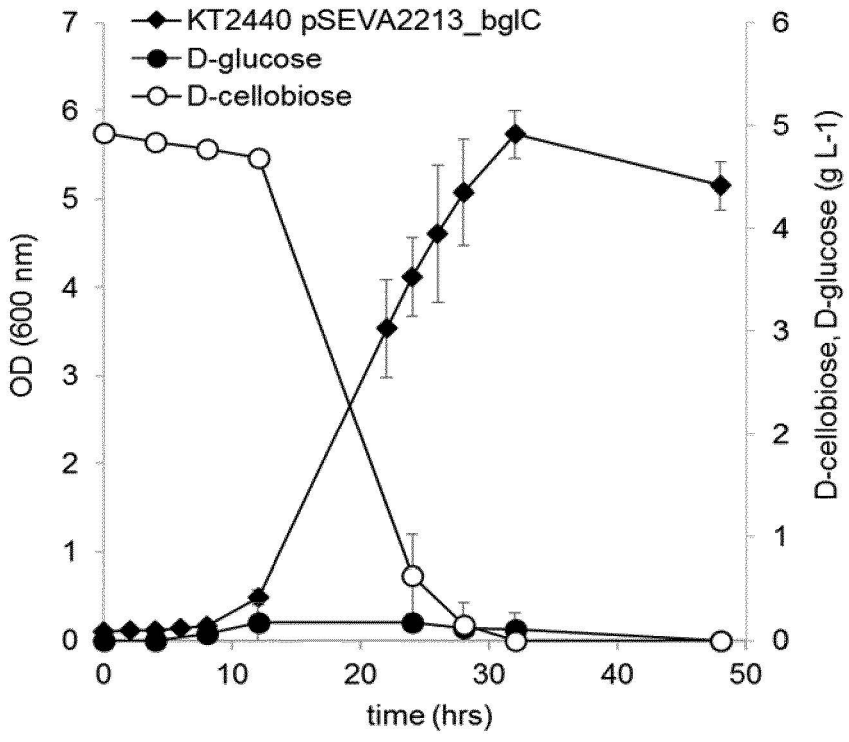


FIG. 9

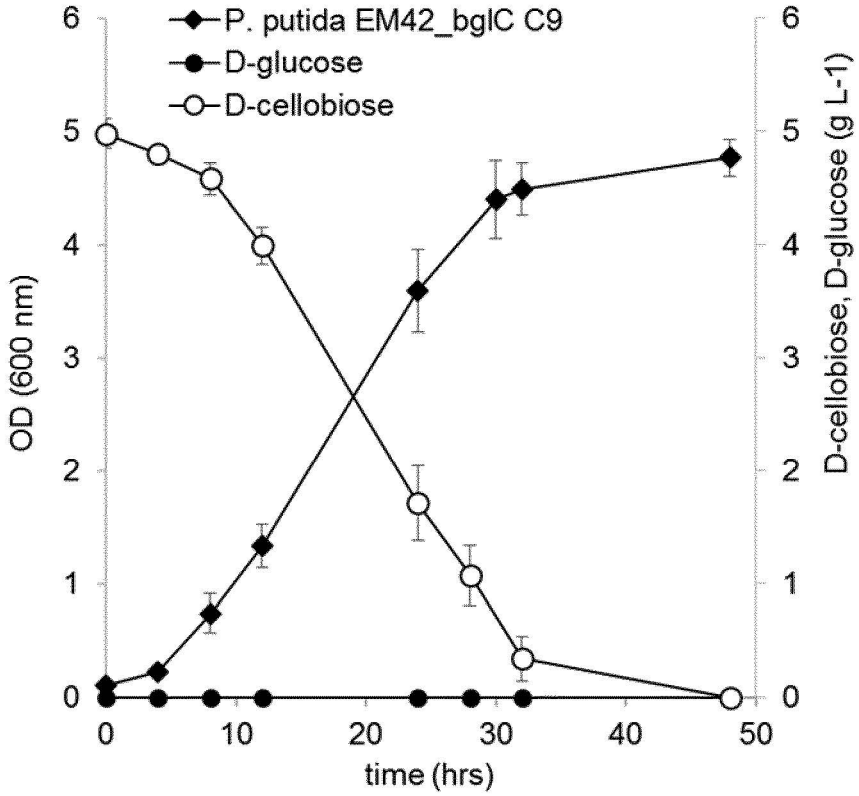

P. putida EM42_bglC C9
D-glucose
D-cellobiose
OD (600 nm)
D-cellobiose, D-glucose (g L-1)
time (hrs)
0
10
20
30
40
50
0
1
2
3
4
5
6

FIG. 10

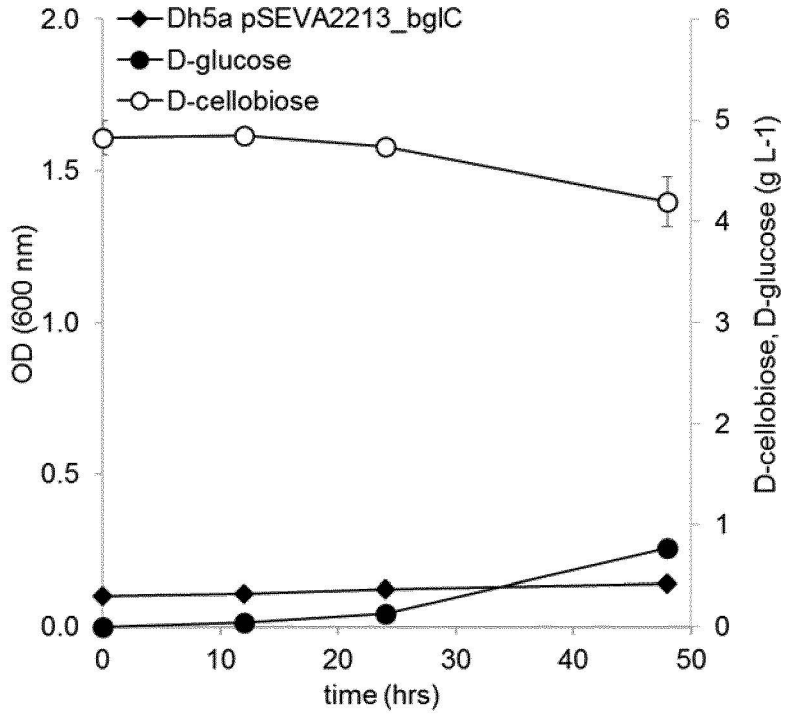
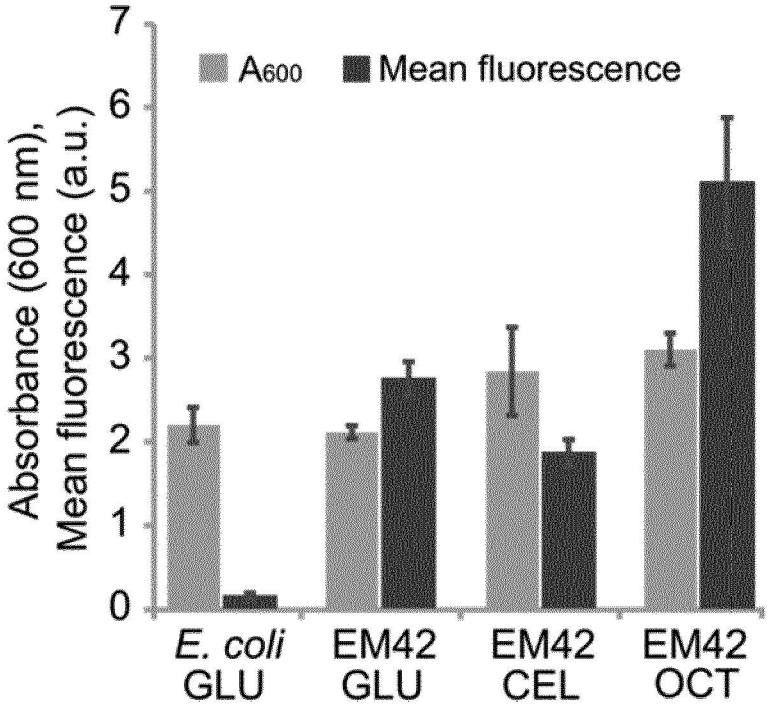

Dh5a pSEVA2213_bglC
D-glucose
D-cellobiose
OD (600 nm)
D-cellobiose, D-glucose (g L-1)
time (hrs)
0
10
20
30
40
50
0.0
0.5
1.0
1.5
2.0
0
1
2
3
4
5
6

FIG. 11


Absorbance (600 nm), Mean fluorescence (a.u.)
0
1
2
3
4
5
6
7
A600
Mean fluorescence
E. coli GLU
EM42 GLU
EM42 CEL
EM42 OCT

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/068347

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/42 C12P7/58 C12P7/62 C12N1/22
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	IASSON E. P. TOZAKIDIS ET AL: "Proof of concept for the simplified breakdown of cellulose by combining Pseudomonas putida strains with surface displayed thermophilic endocellulase, exocellulase and [beta]-glucosidase", MICROBIAL CELL FACTORIES, vol. 15, no. 1, 10 June 2016 (2016-06-10), XP055506359, DOI: 10.1186/s12934-016-0505-8 the whole document -----	1-7
A	US 2016/257980 A1 (LINGER JEFFREY [US] ET AL) 8 September 2016 (2016-09-08) example 7 -----	1-17
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 September 2018	24/09/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lejeune, Robert

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TSUTOMU TANAKA ET AL: "Creation of Cellobiose and Xylooligosaccharides-Coutilizing Escherichia coli Displaying both [beta]-Glucosidase and [beta]-Xylosidase on Its Cell Surface", ACS SYNTHETIC BIOLOGY, vol. 3, no. 7, 8 November 2013 (2013-11-08), pages 446-453, XP055506366, Washington, DC,USA ISSN: 2161-5063, DOI: 10.1021/sb400070q the whole document	1-17
A	NIKOLAY A. SPIRIDONOV ET AL: "Cloning and Biochemical Characterization of BglC, a [beta]-Glucosidase from the Cellulolytic Actinomycete Thermobifida fusca", CURRENT MICROBIOLOGY, vol. 42, no. 4, 1 April 2001 (2001-04-01), pages 295-301, XP055506367, Boston ISSN: 0343-8651, DOI: 10.1007/s002840110220 the whole document	1-17
X	US 2 463 784 A (LOCKWOOD LEWIS B) 8 March 1949 (1949-03-08) table IV	8-15
A	WO 2017/016949 A1 (ANNIKKI GMBH [AT]) 2 February 2017 (2017-02-02) the whole document	8-15
X	WO 2013/072541 A1 (CONSEJO SUPERIOR INVESTIGACION [ES]) 23 May 2013 (2013-05-23) the whole document	16,17
T	DVORÁK PAVEL ET AL: "Refactoring the upper sugar metabolism ofPseudomonas putidafor co-utilization of cellobiose, xylose, and glucose", METABOLIC ENGINEERING, vol. 48, 2 June 2018 (2018-06-02), pages 94-108, XP085437899, ISSN: 1096-7176, DOI: 10.1016/J.YMBEN.2018.05.019 the whole document	1-17

-/--

1

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ESTEBAN MART?NEZ-GARC?A ET AL: "Pseudomonas 2.0: genetic upgrading of P. putida KT2440 as an enhanced host for heterologous gene expression", MICROBIAL CELL FACTORIES,, vol. 13, no. 1, 11 November 2014 (2014-11-11), page 159, XP021221344, ISSN: 1475-2859, DOI: 10.1186/S12934-014-0159-3 the whole document	1-17
A	MERVI H TOIVARI ET AL: "Microbial-xylonate production", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, BERLIN, DE, vol. 96, no. 1, 9 August 2012 (2012-08-09) , pages 1-8, XP035107813, ISSN: 1432-0614, DOI: 10.1007/S00253-012-4288-5 cited in the application the whole document	1-17
A	HARDY G P M A ET AL: "Energy conservation by pyrroloquinoline quinol-linked xylose oxidation in Pseudomonas putida NCTC 10936 during carbon-limited growth in chemostat culture", FEMS MICROBIOLOGY LETTERS, WILEY-BLACKWELL PUBLISHING LTD, GB, vol. 107, no. 1, 15 February 1993 (1993-02-15), pages 107-110, XP023984994, ISSN: 0378-1097, DOI: 10.1111/J.1574-6968.1993.TB06012.X [retrieved on 1993-02-15] the whole document	1-17

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/068347

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2016257980 A1	08-09-2016	US 2016257980 A1	08-09-2016
		US 2017327854 A1	16-11-2017
US 2463784 A	08-03-1949	NONE	
WO 2017016949 A1	02-02-2017	AR 105445 A1	04-10-2017
		CN 107849589 A	27-03-2018
		EP 3325638 A1	30-05-2018
		JP 2018524024 A	30-08-2018
		US 2018216142 A1	02-08-2018
		WO 2017016949 A1	02-02-2017
WO 2013072541 A1	23-05-2013	ES 2423499 A1	20-09-2013
		WO 2013072541 A1	23-05-2013