

Article

Bacterial Whole Cells Synthesis of Whisky Lactones in a Solid-State Fermentation Bioreactor Prototype

Dawid Hernik ^{1,*}, Jakub Pannek ^{1,2}, Ewa Szczepańska ¹, Teresa Olejniczak ¹ and Filip Boratyński ^{1,*}

¹ Department of Chemistry, Wrocław University of Environmental and Life Sciences, Norwida 25, 50-375 Wrocław, Poland; jakub.pannek@rdhub.pl (J.P.); ewa.szczepanska@upwr.edu.pl (E.S.); teresa.olejniczak@upwr.edu.pl (T.O.)

² R&D Hub Spółka Z Ograniczoną Odpowiedzialnością (sp. z o. o.), Spokojna 10, 98-270 Złoczew, Poland

* Correspondence: dawid.hernik@upwr.edu.pl (D.H.); filip.boratynski@upwr.edu.pl (F.B.)

Abstract: Agro-industrial side streams such as oilseed cakes were used as a medium in solid-state fermentation (SSF) for microbial oxidation of *anti*- and *syn*-3-methyl-octane-1,4-diols to obtain corresponding *trans*- and *cis*-whisky lactones. In preliminary screening transformations, a wide range of whole bacterial cells were tested on the basis of oxidation activity, which is rarely described in the literature, in contrast to the widely studied lipolytic activity on SSF. Among the different oil cakes tested, biotransformations carried out on linseed cake were characterized by the highest conversion and stereoselectivity. Several preparative-scale oxidations performed in a self-constructed SSF bioreactor catalyzed by *Rhodococcus erythropolis* DSM44534, *Rhodococcus erythropolis* PCM2150 and *Gordonia rubripertincta* PCM2144 afforded optically active *trans*-(+)-(4*S*,5*R*), *cis*-(+)-(4*R*,5*R*) and *cis*-(-)-(4*S*,5*S*) isomers of whisky lactones, respectively. Bacteria of the *Rhodococcus*, *Gordonia*, *Dietzia* and *Streptomyces* genera carried out transformations with complete conversion after three days. Various extraction methods were applied for the isolation of the products, and among them, the combination of steam distillation with simple extraction were the most efficient. Biotransformations were conducted under precise control of conditions in a bioreactor based on a Raspberry Pi Zero W. The proposed low-cost (ca. USD 100) bioreactor is a standalone system that is fully autoclavable and easy to use.

Keywords: biotransformation; microbial oxidation; whisky lactones; diols; solid-state fermentation (SSF); bioreactor; Raspberry Pi



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1. Introduction

Solid-state fermentation (SSF) is defined as a microbial cultivation process conducted on a solid substrate with a low content of water [1]. Several bioprocesses based on agro-industrial residues, such as the production of enzymes, single-cell proteins, aromas, organic acids, ethanol, biopolymers, and other secondary metabolites, have been developed [2–11]. In both laboratory- and industrial-scale microbial processes, it is essential to control culture conditions such as temperature, moisture content, aeration, and pH [12]. In comparison to submerged fermentation (SmF), monitoring the aforementioned process parameters during SSF is a challenge because of the solid nature of the substrate. However, due to numerous advantages (low cost of growth media, low energy requirement, low contamination risk, low pollutant production, and high isolation yields), it is worth applying SSF in industry. Of note, SSF use low-cost raw materials such as agro-industrial which significantly lowers the capital investment required for particular bioprocesses in comparison to submerged fermentation, in which liquid media are used [13].

In recent years, attention has been given to significantly reducing the generation of food waste. Agro-industrial residues have special biotechnological potential and can be used to solve this problem. They meet the criteria enabling their use as raw materials for the SSF process, i.e., low water content and optimal particle size allowing penetration of the

substrate by microorganisms (especially by filamentous fungi) [14]. Oilseed cakes are solid residues obtained as a result of the pressing of oilseeds during vegetable oil production. They can constitute up to 65–70% of seeds' weight. The oleo industry generates millions of tons of these organic byproducts every year; therefore, focusing on the various processes related to the value addition of oil cakes is needed.

Biotransformation is a process in which microorganisms or isolated enzymes are applied to obtain fine chemicals, such as enantiopure forms of chiral compounds [6,15–17]. Obtaining the desired products by the use of environmentally safe biocatalysts and raw materials makes this approach possible to meet the requirements of sustainable development and green chemistry [18]. Compounds obtained by this method are regarded as natural [19].

The increase in the application of SSF as an alternate production method to SmF by industry has been recently noted [20,21]. Despite the progress made in research on the use of SSF, the main obstacle to scaling up the process is the lack of simple, economical and easily scalable bioreactors that eliminate the problem of controlling the process conditions. The basic SSF bioreactors are tray, horizontal drum, packed-bed, and fluidized bed bioreactors, where tray bioreactors have the simplest construction and process performance. The approach proposed in this study constitutes the application of whole-cell biotransformation on oilseed cake as a medium for microorganisms using an upscaled tray bioreactor with sensors controlling the basic culture conditions (temperature and moisture). Due to a number of SSF applications, a new bioprocess involving bacterial oxidoreductases to produce industrially demanded whisky lactone was developed.

Whisky lactone is an essential component of aged alcoholic beverages such as whisky, cognac, and brandy beverages [22]. Presently, it is used as an odor ingredient in various food products (beverages and sweet and baked foods). This commercial food additive contains a racemic mixture of *trans*- and *cis*-isomers. However, in nature, individual *trans*-(+)-(4*S*,5*R*) and *cis*-(+)-(4*S*,5*S*) isomers of whisky lactone, which exhibit individual odoriferous properties, occur [23,24]. *Cis*-isomers are described as earthy and woody fragrances, while *trans*-isomers are reminiscent of celery. Therefore, the manufacture of individual stereoisomers of whisky lactone to study the relationship between its structure and biological activity is necessary. Whisky lactone is not only an aroma; the mixture of *cis*- and *trans*-isomers is used as a repellent against mosquitoes and flies [25].

Several stereoselective pathways for the synthesis of individual *trans*- and *cis*-isomers of whisky lactone have been presented in the literature [14,26–29]. However, they rely on a multistep chemical synthesis using metal catalysts and organic solvents. Catalysis involving metal compounds is widely engaged in the laboratory and industrial practice. Nevertheless, metal-based catalysts as well as organic solvents also can be dangerous for environment, and significant amounts of them are released to environment annually. The employment of biocatalysts in organic synthesis is seen as the key to green chemistry. It is worth mentioning that the method of obtaining whisky lactone isomers using a non-metallic catalyst has also been described [30]. This process is promising because it is an alternative to metal catalysts, however, it is a multi-step process with a relatively low conversion. Therefore, there is a need for the development of safe procedures that fulfills green chemistry requirements to obtain stereoisomers. To date, two pathways of biotransformation have been proposed: *via* alcohol dehydrogenase isolated from horse liver (HLADH) enantioselectivity oxidizing racemic *syn*- and *anti*-3-methyloctane-1,4-diols as well as with the use of *Beauveria bassiana* AM278 and *Pycnidium resinae* KCH50 microorganisms catalyzing lactonization of γ -oxo acids. The application of the second strategy allowed us to obtain enantiomerically pure *trans*-(+)-(4*S*,5*R*)-whisky lactone [31].

The study aimed to perform the oxidation of *anti*-3-methyl-octane-1,4-diol (**1a**) and *syn*-3-methyl-octane-1,4-diol (**1b**) catalyzed by bacteria growing on oilseed cakes. It is worth mentioning that the SSF processes involving oxidation on a solid substrate are not commonly known in the literature. To date, microbial oxidation of xenobiotics performed on oil industry byproducts has been reported with the use of filamentous fungi, which cat-

alyze stereoselective hydrolysis of a racemic mixture of phenylethyl acetate and subsequent oxidation of 1-phenylethanol to acetophenone [32]. Research on reprocessing waste often requires very large financial contributions, including research equipment. In this article, we described a low-cost bioreactor with a potential environmental impact. To increase the efficiency of whisky lactone production, monitoring of cultivation parameters in the prototype bioreactor was performed.

2. Results and Discussion

Substrates for biooxidation, *anti*- (**1a**) and *syn*-3-methyl-octane-1,4-diols (**1b**), were obtained by chemical reduction of corresponding *trans*- (**2a–b**) and *cis*-whisky lactones (**2c–d**), which were previously separated by column chromatography (Figure 1). On the basis of our previous experience in the biooxidation reactions [33–35], four bacterial strains (*Gordonia bronchialis* PCM2167, *Rhodococcus ruber* PCM2166, *Rhodococcus erythropolis* DSM44534 and *Rhodococcus rhodochrous* PCM909) were selected as potential candidates for microbial oxidation of *anti*- (**1a**) and *syn*-3-methyl-octane-1,4-diols (**1b**) to obtain corresponding *trans*-(+)-(4*S*,5*R*) (**2a**) and *trans*-(-)-(4*R*,5*S*) (**2b**) or *cis*-(-)-(4*S*,5*S*) (**2c**) and *cis*-(+)-(4*R*,5*R*) (**2d**) whisky lactones (Figure 2). The same microorganisms were also used in our recent studies (pending patent protection), in which 3-*n*-butylphthalide was obtained as a result of oxidation of the corresponding diol (1-hydroxymethyl-2-(1-hydroxypentyl)benzene). Bacteria from selected species were characterized by high activity of alcohol dehydrogenases (ADH) responsible for one-pot oxidation of diols to lactones which was confirmed in our previous studies [33–35].

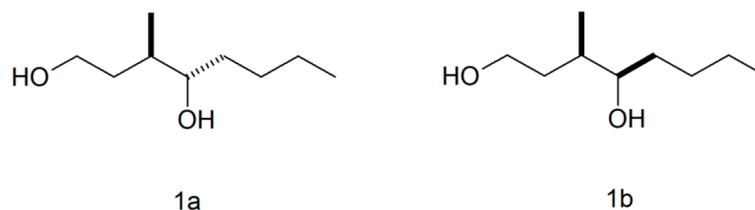


Figure 1. Structures of *anti*- (**1a**) and *syn*-3-methyl-octane-1,4-diols (**1b**).

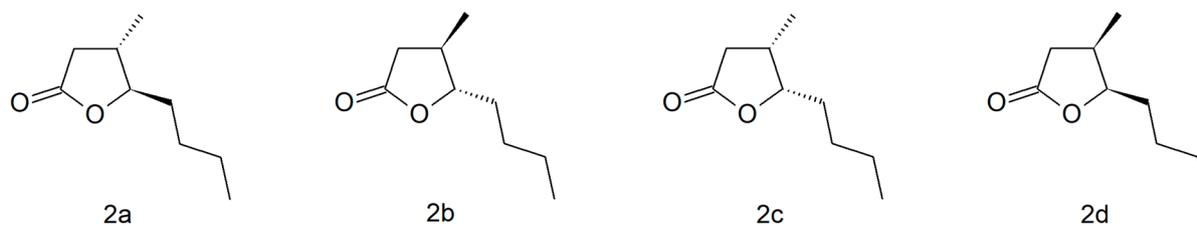


Figure 2. Structures of *trans*-(+)-(4*S*,5*R*) (**2a**), *trans*-(-)-(4*R*,5*S*) (**2b**), *cis*-(-)-(4*S*,5*S*) (**2c**) and *cis*-(+)-(4*R*,5*R*) (**2d**) whisky lactones.

The growth of biocatalysts as well as the biotransformation process was performed *via* solid-state fermentation using three different oleoindustry byproducts: linseed, rapeseed and evening primrose cakes. These residues are abundant in nutritional compounds such as carbohydrates, proteins, fats, and cellulose and therefore constitute excellent media for the growth of microorganisms [36,37]. The average moisture of the studied oil cakes (7.9% for linseed cake, 6.5% for rapeseed cake and 4.2% for evening primrose cake) was calculated by the lyophilization of individual oil cake samples. This confirmed our observation, during which the linseed cake had the highest hygroscopic properties, while the evening primrose cake absorbed water slightly.

2.1. Preliminary Screening Scale Biotransformations with *Anti*-3-methyl-octane-1,4-diol (**1a**) on Different Oil Cakes

In the oxidation of *anti*-3-methyl-octane-1,4-diol (**1a**) with all tested strains growing on the linseed cake, the complete conversion (*conv.* = 100%) after three days was observed

(Table 1). Even though *cis*-whisky lactone was obtained in enantiopure form (*ee* > 99%) in all biotransformations, enantiomerically enriched *trans*-whisky lactone (*ee* = 20–70%) was a dominant product.

Table 1. Comparison of microbial oxidation of *anti*-3-methyl-octane-1,4-diol (**1a**) on linseed and rapeseed cake (in % according to GC).

Strain	Oil Cake	Time [days]	Conv. 1a [%]	Products			
				<i>Trans</i> 2a–b [%]	<i>ee</i> [%]	<i>Cis</i> 2c–d [%]	<i>ee</i> [%]
<i>Gordonia bronchialis</i> PCM2167	linseed	3	100	83 (±0.7)	33 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	17 (±1.5)	>99 (–)-(4 <i>S</i> ,5 <i>S</i>)- 2c
		7	100	81 (±1.2)	33 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	19 (±1.3)	>99 (–)-(4 <i>S</i> ,5 <i>S</i>)- 2c
	rapeseed	3	9 (±0.9)	7 (±0.4)	32 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	2 (±0.1)	0
		7	12 (±1.1)	10 (±0.8)	18 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	2 (±0.1)	0
<i>Rhodococcus erythropolis</i> DSM44534	linseed	3	100	97 (±1.2)	20 (–)-(4 <i>R</i> ,5 <i>S</i>)- 2b	3 (±0.2)	>99 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
		7	100	90 (±0.9)	70 (–)-(4 <i>R</i> ,5 <i>S</i>)- 2b	10 (±0.5)	>99 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	rapeseed	3	15 (±0.7)	8 (±0.7)	nd*	7 (±0.2)	nd
		7	43 (±2.1)	9 (±0.8)	nd	34 (±1.2)	nd
<i>Rhodococcus rhodochrous</i> PCM909	linseed	3	100	92 (±0.7)	20 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	8 (±0.3)	>99 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
		7	100	90 (±0.9)	42 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	10 (±0.4)	>99 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	rapeseed	3	0	0	0	0	0
		7	0	0	0	0	0
<i>Rhodococcus ruber</i> PCM2166	linseed	3	100	92 (±1.3)	37 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	8 (±0.1)	>99 (–)-(4 <i>S</i> ,5 <i>S</i>)- 2c
		7	100	86 (±0.6)	33 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	14 (±0.8)	65 (–)-(4 <i>S</i> ,5 <i>S</i>)- 2c
	rapeseed	3	0	0	0	0	0
		7	84 (±0.6)	67 (±1.2)	5 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	17 (±1.2)	0

nd*—not determined.

Oxidation with *Gordonia bronchialis* PCM2167 on linseed cake afforded optically active *trans*-(+)-(4*S*,5*R*)-isomer (**2a**) (81–83%, *ee* = 33%) and enantiomerically pure *cis*-(–)-(4*S*,5*S*) whisky lactone (**2c**) (17–19%, *ee* >99%). In biotransformations performed on linseed cake with *Rhodococcus erythropolis* DSM44534, *R. rhodochrous* PCM909 and *R. ruber* PCM2166, optically active *trans*-whisky lactone isomers were obtained after three days in amounts of 97%, 92% and 92%, respectively. It is significant that after 7 days, *R. erythropolis* DSM44534 produced *trans*-(–)-(4*R*,5*S*)-isomer (**2b**) (*ee* = 70%), while *R. ruber* PCM2166 and *R. rhodochrous* PCM909 gave the opposite *trans*-(+)-(4*S*,5*R*)-isomer (**2a**) (*ee* = 33–42%). The enantioselectivity of biotransformation increases over time; thus, the enantiomeric excess of lactone was higher after 7 days. On the other hand, transformation catalyzed by *G. bronchialis* PCM2167 afforded higher amounts (*ca.* 20%) of enantiomerically pure *cis*-(–)-(4*S*,5*S*) whisky lactone (**2c**) compared with *Rhodococcus* strains.

A very low conversion (9 to 43%) for *G. bronchialis* PCM2167 and *R. erythropolis* DSM44534, except oxidation with *R. ruber* PCM2166 (84% after 7 days), was observed in biotransformations performed on rapeseed cake. No conversion was observed during the biotransformation with *R. ruber* PCM2166 after three days which indicates that alcohol dehydrogenases are produced by this strain in the later stages of biotransformation. In the biooxidation with *R. rhodochrous* PCM909, no conversion on the rapeseed cake occurred. This indicates that rapeseed cake is an inadequate medium for ADH production by aforementioned biocatalyst. No whisky lactones were detected by using primrose cake for solid-state fermentation; thus, this byproduct is not applicable for diol oxidation.

2.2. Preliminary Screening Scale Biotransformations with *Syn*-3-methyl-octane-1,4-diol (**1b**) on Different Oil Cakes

During the oxidation of *syn*-3-methyl-octane-1,4-diol (**1b**) on the linseed cake with all bacterial strains, 100% conversion was observed after the 3rd day (Table 2). Although

the transformations catalyzed by *Gordonia bronchialis* PCM2167, *Rhodococcus rhodochrous* PCM909 and *R. ruber* PCM2166 afforded enantiomerically pure *trans*-(+)-(4*S*,5*R*) whisky lactone (*ee* >99%), enantiomerically enriched *cis*-(+)-(4*R*,5*R*) isomer (**2d**) constituted the majority of the reaction mixture (63–81%). Biotransformation with *G. bronchialis* PCM2167 on linseed cake after three days gave *trans*-(+)-(4*S*,5*R*) enantiomer (**2a**) (19%, *ee* >99%) and enantiomerically enriched *cis*-(+)-(4*R*,5*R*) whisky lactone (**2d**) (83%, *ee* = 93%). In the biotransformations with *G. bronchialis* PCM2167, it was observed that from days 3 to 7, the enantiomeric excess of *cis*-(+)-(4*R*,5*R*) (**2d**) isomer decreased from *ee* = 93% to 85%.

Table 2. Comparison of microbial oxidation of *syn*-3-methyl-octane-1,4-diol (**1b**) on linseed and rapeseed cake (in % according to GC).

Strain	Oil Cake	Time [days]	Conv. 1b [%]	Products			
				<i>Trans</i> 2a–b [%]	<i>ee</i> [%]	<i>Cis</i> 2c–d [%]	<i>ee</i> [%]
<i>Gordonia bronchialis</i> PCM2167	linseed	3	100	19 (±0.7)	>99 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	81 (±0.7)	93 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
		7	100	21 (±1.3)	>99 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	79 (±1.7)	85 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	rapeseed	3	0	0	0	0	0
		7	12 (±0.9)	10	0	2 (±0.4)	82 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
<i>Rhodococcus erythropolis</i> DSM44534	linseed	3	100	27 (±0.9)	37 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	73 (±1.0)	83 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
		7	100	23 (±0.6)	78 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	77 (±1.1)	86 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	rapeseed	3	18 (±0.7)	4 (±0.1)	0	14 (±0.3)	0
		7	57 (±1.5)	9 (±0.3)	0	48 (±1.6)	4 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
<i>Rhodococcus rhodochrous</i> PCM909	linseed	3	100	25 (±0.9)	>99 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	75 (±1.2)	65 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
		7	100	31 (±0.4)	>99 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	69 (±0.7)	67 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	rapeseed	3	0	0	0	0	0
		7	0	0	0	0	0
<i>Rhodococcus ruber</i> PCM2166	linseed	3	100	37 (±0.9)	>99 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	63 (±1.6)	79 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
		7	100	37 (±1.2)	>99 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	63 (±0.8)	83 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	rapeseed	3	0	0	0	0	0
		7	22 (±0.7)	6 (±0.3)	0	16 (±0.9)	0

On the rapeseed cake, the low conversion of diol and only small amounts of both *trans*- and *cis*-whisky lactone isomers were observed. The highest conversion of *syn*-3-methyl-octane-1,4-diol (**1b**) was observed on rapeseed cake with *R. erythropolis* DSM44534. There was no conversion in transformation with *R. rhodochrous* PCM909 on rapeseed cake, likewise in oxidation with *anti*-3-methyl-octane-1,4-diol (**1a**). In biotransformations on primrose oilcake, no conversion of *syn*-3-methyl-octane-1,4-diol (**1b**), similar to that of *anti*-3-methyl-octane-1,4-diol (**1a**), was detected.

In biotransformations performed on the linseed cake with *G. bronchialis* PCM2167, *R. ruber* PCM2166, *R. erythropolis* DSM44534 and *R. rhodochrous* PCM909 (2.1 and 2.2), significantly more *trans* whisky lactone (81–97%) was formed when *anti*-3-methyl-octane-1,4-diol (**1a**) was used as a substrate (Table 1). On the other hand, when *syn*-3-methyl-octane-1,4-diol (**1b**) was applied, *cis*-isomer (63–81%) was formed predominantly (Table 2). Among tested microorganisms it was noticed that in transformations with *R. ruber* PCM2166 on rapeseed cake, higher conversion (84%) was obtained with **1a** compared to **1b** (22%)."

According to our previous results [31], *trans*-(+)-(4*S*,5*R*) (**2a**) and *cis*-(-)-(4*S*,5*S*) (**2c**) enantiomers of whisky lactones were produced as a result of microbial whole-cell lactonization of the corresponding γ -oxoacids. The opposite enantiomerically enriched *trans*-(-)-(4*R*,5*S*) (**2b**) and *cis*-(+)-(4*R*,5*R*) (**2d**) whisky lactones were obtained in enzymatic oxidation catalyzed by commercially available alcohol dehydrogenases. We developed a SSF oxidation process as an interesting alternative against redox reactions catalyzed by expensive enzymes and required coenzymes.

There are two possible approaches to obtain chiral lactones from racemic diols [33]. In the first one (Figure 3), the primary hydroxy group of diol is chemoselectively oxidized to a carboxylic group, and then, the corresponding hydroxy carboxylic acid is cyclized to a lactone product. However, in the second pathway, there is a two-step oxidation process. First, diol is oxidized to the corresponding hydroxyaldehyde; then, hemiacetal is formed spontaneously, and it is further oxidized to lactone. Our previous studies, due to isolated hemiacetals, confirmed the second pathway of enzymatic oxidation of diols to whisky lactones. However, in the second pathway, there is a two-step oxidation process. First, diol is oxidized to the corresponding hydroxyaldehyde; then, hemiacetal is formed spontaneously, and it is further oxidized to lactone. Our previous studies, due to isolated hemiacetals, confirmed the second pathway of enzymatic oxidation of diols to whisky lactones [33].

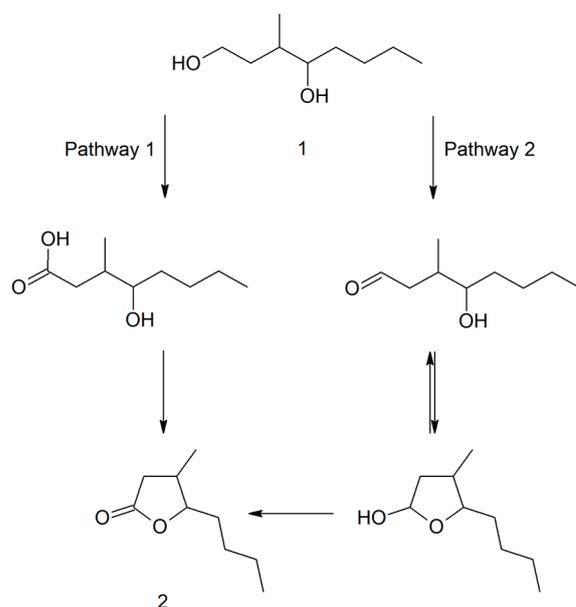


Figure 3. Two possible pathways of biotransformation of 3-methyl-octane-1,4-diol (1).

On the basis of our research, we cannot clearly confirm which of the abovementioned mechanisms takes place during the oxidation catalyzed by bacterial cells. As a result of bacterial oxidation of the diols (1a and 1b) in the preliminary screening, we obtained enantiomerically pure or enriched isomers opposite to those isolated in our previous research [31,33]. Therefore, further research is required to confirm which path is actually involved in this process because no intermediate products were isolated.

2.3. Preliminary Screening Scale Biotransformations with a Diastereoisomeric Mixture of Anti- and Syn-3-methyl-octane-1,4-diols (1a–b)

On the basis of previous screening, only linseed cake was selected for these studies. It is supposed to be related to facts that the linseed cake is characterized by the highest ability to absorb water [37]. In the case of bacteria, it is of great importance because they grow in the entire volume of the substrate, and the even distribution of water throughout the growth medium also causes even bacterial growth. In the case of evening primrose cake, only a part of the water is absorbed, and the rest is on the surface of the substrate, which can reduce bacterial growth. In addition, evening primrose oilcake contains much more fiber, which can slow down the growth of bacteria because it makes them less accessible to nutrients [38]. Rapeseed cake contains sinapic acid, which has antimicrobial activity therefore probably causes worse bacterial growth on this byproduct [39].

Biotransformations with all tested strains with a diastereoisomeric mixture of *anti*- and *syn*-3-methyl-octane-1,4-diols (1a–b) were performed to determine how the mixture

of substrate stereoisomers affects the obtained products concerning the conversion and enantiomeric purity of lactones (Table 3). The results obtained from the oxidation of a mixture of *anti*- and *syn*-diols were the resultant of those received in individual biotransformations presented in paragraphs 2.1 and 2.2. In most biotransformations the *trans*/*cis* ratio of whisky lactone isomers was in the range of 39–60% for *trans*-**2a** 40–61% for *cis*-**2d** isomer. Among tested strains the ratio of formed *trans*/*cis*-whisky lactones differs significantly only in the transformation with *Gordonia bronchialis* PCM2167 after three days affording 23% of the *trans*-isomer **2a** and 77% of the *cis*-isomer **2d**. However, after seven days it was 45/55%, similar ratio to the rest of the biotransformations. The highest enantiomeric excess (*ee* = 90%) of *cis*-(+)-(4*R*,5*R*) lactone (**2d**) was obtained in the *G. bronchialis* PCM2167 culture after three days. In all biotransformations the conversion was 100%. Since a diastereoisomeric mixture of diols was used as a substrate, a significant decrease in the biotransformation enantioselectivity was observed; thus, separate biotransformations with the individual isomers in subsequent studies were subsequently performed.

Table 3. Microbial oxidation of a mixture of *anti*- and *syn*-3-methyl-octane-1,4-diols (**1a–b**) on linseed cake (in % according to GC).

Strain	Time [days]	Conv. 1a–1b [%]	Products			
			<i>Trans</i> 2a–b [%]	<i>ee</i> (+)-(4 <i>S</i> ,5 <i>R</i>)-2a [%]	<i>Cis</i> 2c–d [%]	<i>ee</i> (+)-(4 <i>R</i> ,5 <i>R</i>)-2d [%]
<i>Gordonia bronchialis</i> PCM2167	3	100	23 (±0.9)	55	77 (±1.2)	90
	7	100	45 (±0.7)	52	55 (±0.6)	70
<i>Rhodococcus erythropolis</i> DSM44534	3	100	47 (±1.1)	32	53 (±1.4)	77
	7	100	47 (±0.4)	35	53 (±0.7)	79
<i>Rhodococcus rhodochrous</i> PCM909	3	100	39 (±0.8)	70	61 (±1.1)	60
	7	100	47 (±1.7)	76	53 (±1.9)	74
<i>Rhodococcus ruber</i> PCM2166	3	100	58 (±1.4)	30	42 (±1.1)	32
	7	100	60 (±1.7)	37	40 (±0.3)	35

2.4. Screening Scale Biotransformations with Anti-3-methyl-octane-1,4-diol (**1a**) on Linseed Cake

During the first round of biotransformations performed on three different oil cakes, only decent amounts of optically pure isomers of whisky lactones were obtained. Therefore, further screening studies were conducted to select bacteria with significant dehydrogenase activity that effectively catalyze the oxidation of diols to lactones. Based on previous experiments, linseed cake was selected as the growth medium for biotransformation. Next, several strains of bacteria from the *Gordonia*, *Rhodococcus*, *Micrococcus*, *Dietzia* and *Streptomyces* species were selected to test their oxidation activity (Table 4).

In transformations carried out with *Gordonia rubripertincta* PCM2144, *Rhodococcus erythropolis* PCM2150, *Rhodococcus coprophilus* PCM2174, *Dietzia* sp. DSM44016 and *Streptomyces griseus subsp. griseus* PCM2331, after three days, *trans*-whisky lactones in amounts ranging from 78% to 100% were produced. The highest enantiomeric excess of *trans*-(+)-(4*S*,5*R*) isomer **2a** (*ee* = 78%) was detected on the 3rd day of transformations with *G. rubripertincta* PCM2144. As a result of oxidation, the *cis*-isomer was obtained with the strains *R. erythropolis* PCM2150, *R. coprophilus* PCM2174, *Dietzia* sp. DSM44016 and *S. griseus subsp. griseus* PCM2331. The enantiomerically pure *cis*-(-)-(4*S*,5*S*) whisky lactone (**2c**) (*ee* > 99%) was obtained in the culture of *R. erythropolis* PCM2150.

By comparing the same genus of bacteria (*G. bronchialis* PCM2167 vs *G. rubripertincta* PCM2144, *R. erythropolis* DSM44534 vs *R. erythropolis* PCM2150, *R. ruber* PCM2171 vs *R. ruber* PCM2216) used in both screening experiments (Tables 2 and 4), there is a similarity in the biotransformations carried out by the strains of the same type.

Table 4. Microbial oxidation of *anti*-3-methyl-octane-1,4-diol (**1a**) on linseed cake (in % according to GC).

Strain	Time [days]	Conv. 1a [%]	Products			
			<i>Trans</i> 2a–b [%]	<i>ee</i> [%]	<i>Cis</i> 2c–d [%]	<i>ee</i> [%]
<i>Dietzia</i> sp. DSM44016	3	100	80 (±1.1)	31 (–)-(4 <i>R</i> ,5 <i>S</i>)- 2b	20 (±0.6)	84 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	7	100	82 (±0.8)	44 (–)-(4 <i>R</i> ,5 <i>S</i>)- 2b	18 (±0.7)	85 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
<i>Gordonia rubripertincta</i> PCM2144	3	100	100	78 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	0	0
	7	100	100	62 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	0	0
<i>Micrococcus luteus</i> PCM525	3	20 (±0.5)	20 (±0.7)	12 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	0	0
	7	25 (±0.9)	25 (±0.2)	15 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	0	0
<i>Rhodococcus coprophilus</i> PCM2174	3	100	95 (±0.6)	7 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	5 (±0.1)	20 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	7	100	93 (±0.5)	3 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	7 (±0.7)	30 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
<i>Rhodococcus erythropolis</i> PCM2150	3	100	80 (±1.1)	0	20 (±0.9)	99 (–)-(4 <i>S</i> ,5 <i>S</i>)- 2c
	7	100	82 (±0.8)	27 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	18 (±0.7)	50 (–)-(4 <i>S</i> ,5 <i>S</i>)- 2c
<i>Rhodococcus ruber</i> PCM2171	3	30 (±0.2)	30 (±0.6)	30 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	0	0
	7	80 (±1.1)	80 (±1.5)	9 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	0	0
<i>Rhodococcus ruber</i> PCM2216	3	0	0	0	0	0
	7	35 (±0.9)	35 (±0.3)	10 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	0	0
<i>Streptomyces griseus</i> subsp. <i>griseus</i> PCM2331	3	100	78 (±1.5)	50 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	22 (±0.7)	8 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	7	100	85 (±1.2)	50 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	15 (±0.4)	25 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d

2.5. Screening Scale Biotransformations with *Syn*-3-methyl-octane-1,4-diol (**1b**) on Linseed Cake

Analysis of the oxidation performed with *G. rubripertincta* PCM2144, *R. erythropolis* PCM2150, *R. coprophilus* PCM2174 and *Dietzia* sp. DSM44016 showed 100% conversion of substrate after three days. During oxidation with most of the strains, enantiomerically enriched *cis*-(+)-(4*R*,5*R*) whisky lactone was obtained (Table 5). The highest amounts from 77% to 100% of this isomer were obtained when *G. rubripertincta* PCM2144, *R. erythropolis* PCM2150, *R. coprophilus* PCM2174 and *Dietzia* sp. DSM44016 were used as the biocatalyst. In the oxidation with *Dietzia* sp. DSM44016 *cis*-(+)-(4*R*,5*R*) isomer was obtained on the 7th day (77%, *ee* = 79%). As a result of transformation, an optically active *trans*-isomer (*ee* = 50–77%) was obtained with *G. rubripertincta* PCM2144, *R. coprophilus* PCM2174 and *Dietzia* sp. DSM44016. Biotransformations with *S. griseus* subsp. *griseus* PCM2331 showed low conversion with a solely small amounts of *trans*- and *cis*-isomers (5–16%).

Table 5. Microbial oxidation of *syn*-3-methyl-octane-1,4-diol (**1b**) on linseed cake (in % according to GC).

Strain	Time [days]	Conv. 1b [%]	Products			
			<i>Trans</i> 2a–b [%]	<i>ee</i> [%]	<i>Cis</i> 2c–d [%]	<i>ee</i> [%]
<i>Dietzia</i> sp. DSM44016	3	100	27 (±0.7)	77 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	73 (±1.2)	75 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	7	100	23 (±0.9)	50 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	77 (±0.4)	79 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
<i>Gordonia rubripertincta</i> PCM2144	3	100	40 (±0.3)	77 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	60 (±1.2)	32 (–)-(4 <i>S</i> ,5 <i>S</i>)- 2c
	7	100	16 (±0.5)	62 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	84 (±2.1)	30 (–)-(4 <i>S</i> ,5 <i>S</i>)- 2c
<i>Micrococcus luteus</i> PCM525	3	30 (±0.4)	0	0	30 (±0.9)	20 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	7	35 (±1.3)	0	0	35 (±1.1)	29 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
<i>Rhodococcus coprophilus</i> PCM2174	3	100	15 (±0.4)	60 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	85 (±1.2)	33 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	7	100	30 (±0.9)	75 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	70 (±0.7)	33 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
<i>Rhodococcus erythropolis</i> PCM2150	3	100	0	0	100	5 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	7	100	0	0	100	2 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
<i>Rhodococcus ruber</i> PCM2171	3	30 (±0.2)	0	0	30 (±1.7)	10 (–)-(4 <i>S</i> ,5 <i>S</i>)- 2c
	7	60 (±1.3)	0	0	60 (±1.8)	0
<i>Rhodococcus ruber</i> PCM2216	3	35 (±0.7)	0	0	35 (±1.1)	30 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2c
	7	35 (±0.4)	0	0	35 (±1.3)	0
<i>Streptomyces griseus</i> subsp. <i>griseus</i> PCM2331	3	10 (±0.2)	5	0	5 (±0.3)	60 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d
	7	27 (±1.2)	16	0	11 (±0.3)	60 (+)-(4 <i>R</i> ,5 <i>R</i>)- 2d

Comparing the same bacteria from *Gordonia* genus only with *G. bronchialis* PCM2167, enantiomerically pure *trans*-whisky lactone was formed (Table 2). *R. erythropolis* PCM2150 (Table 5) transformed diol (**1b**) to *cis*-whisky lactone with lower enantiomeric excess than *R. erythropolis* DSM44534 (Table 2), and no *trans*-isomer was formed. *R. ruber* PCM2166 (Table 2) oxidized diol (**1b**) with higher conversion and enantioselectivity than *R. ruber* PCM2171 and *R. ruber* PCM2216 (Table 5).

During biotransformation of *anti*- and *syn*-diols with strains *Dietzia maris* PCM2292 and *Rhodococcus rhodnii* PCM2157 (not mentioned in Tables 4 and 5), there was no conversion, which proves that these bacteria do not have the ability to oxidize 3-methyl-octane-1,4-diol on linseed cake. Biotransformations with *Micrococcus luteus* PCM525 and *Rhodococcus ruber* PCM2216 showed low conversion (20–35%). With *anti*-diol (**1a**), only a small amount of *trans*-isomer (20–35%) was formed, and with *syn*-diol (**1b**) only *cis*-isomer of whisky lactone was produced (30–35%).

In biooxidation carried out with the following strains *Dietzia* sp. DSM44016, *G. rubripertincta* PCM2144, *R. coprophilus* PCM2174, *R. erythropolis* PCM2150, *R. ruber* PCM2171, and *S. griseus* subsp. *griseus* PCM2331 (Tables 4 and 5), it was also noticed that more *trans*-whisky lactone was produced after seven days (80–100%) when the *anti*-diol (**1a**) was used as the substrate. However, when *syn*-diol (**1b**) was added, more *cis*-whisky lactone isomer was formed predominantly after seven days (60–100%) within the same strains, except for *S. griseus* subsp. *griseus* PCM2331 (5–11%). We identified three biocatalysts *M. luteus* PCM525, *R. ruber* PCM2171, *R. ruber* PCM2216, which catalyzed highly diastereoselective oxidation independently of the substrate used, affording only *trans*-whisky isomers from *anti*-diol and *cis*-whisky isomers from *syn*-diol. Application of two stereochemically different substrates in biotransformations catalyzed by *G. rubripertincta* PCM2144 and *R. erythropolis* PCM2150 was significant concerning diastereoselectivity of the process. In oxidation catalyzed by *G. rubripertincta* PCM2144 using *anti*-diol only *trans*-isomer was obtained, however with *syn*-diol a mixture of *cis*/*trans*-isomers was observed. In contrary, *R. erythropolis* PCM2150 catalyzed oxidation of *syn*-diol to only *cis*-lactone, while from *anti*-diol a mixture of *cis*/*trans*-whisky isomers was detected.

2.6. Selection of the Extraction Method

On the basis of screening studies, a simple extraction method using organic solvents was applied to isolate biotransformation products on a preparative scale. While conducting simple extraction in preparative biotransformations, a problem arose related to the extraction of excessive amounts of fat from linseed cake. Attempts to separate the lipid fraction *via* column chromatography were unsuccessful; the columns clogged, and only 10–15% of the product was obtained. Therefore, it was decided to test other extraction methods. Extraction with the Deryng apparatus was then tested, but the cake foamed and burned easily when the flask was heated (even at low heating temperatures). In next method steam distillation was tested. The efficiency during steam distillation was 85%; however, due to the high content of protein in the cake, it foamed considerably. Therefore, the extraction process had to be performed several times because the resulting foam filled the entire distillation flask, which significantly extended the time of this method. Taking into consideration this fact, it was decided to conduct steam distillation from organic extract first to obtain a simple extraction of the preparative biotransformation. The yield of this method was 81%, and distillation could be performed in one step due to the volume of the obtained extract was small and did not foam. This method turned out to be the most effective among all tested and was used in all preparative biotransformations.

2.7. Calibration of Moisture Sensor

To control humidity during biotransformation in the bioreactor, two types of electrodes were tested. First, measurements were carried out with graphite electrodes and then with a probe for measuring soil moisture (Waveshare 9527) on linseed and rapeseed cake. Based on measurements conducted with graphite electrodes, a correlation graph of moisture and

voltage in rapeseed cakes was created (Figure 4a). The lowest value of moisture was 15% for rapeseed cake. With moisture lower than 15%, the sensor could not detect voltage. The voltage increased for a moisture from 15 to 90%. Figure 4b shows that for linseed cake, the lowest value of moisture conducted with graphite electrodes, which could be marked, was 20%. Voltage increased for the moisture from 20 to 90%. A steady increase in voltage was observed for moisture in the range from 40 to 50%. The smallest increase in voltage was observed from 60 to 70% moisture, and the largest increase was from 30 to 60% moisture.

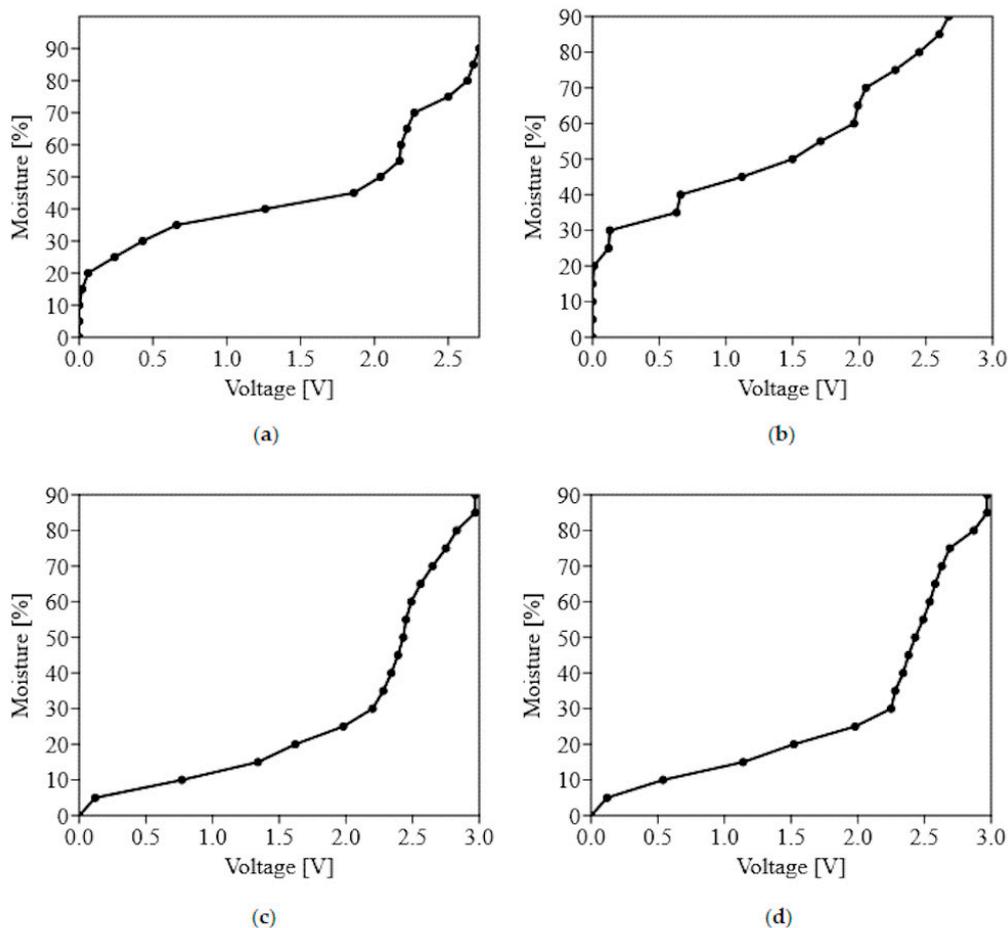


Figure 4. Correlation curves of moisture and voltage: (a) measured with graphite electrodes for rapeseed cake; (b) measured with graphite electrodes for linseed cake; (c) measured with probe (Waveshare 9527) for rapeseed cake; (d) measured with probe (Waveshare 9527) for linseed cake.

When measuring the moisture in the rapeseed cake with a probe (Waveshare 9527), the first measurable voltage reading was 5% medium moisture (Figure 4c). The maximum reading range ended at 85% medium moisture. In the ranges of 5% to 30%, 30% to 60% and 60% to 80%, the values were similar to linear, allowing humidity control between these values. The first measurement at 5% humidity was also obtained in the measurement of moisture on the linseed cake using a probe with a humidity sensor (Waveshare 9527). The maximum range also ended at 85% humidity because the voltage was constant above this value (Figure 4d). Linear values were obtained in the range from 5 to 30% and from 30 to 75% humidity, allowing for the precise control of humidity in the medium.

Comparing the values obtained when controlling the medium with different electrodes, we decided to use a sensor with a probe to measure the humidity. This made it possible to obtain more linear values in wide ranges of humidity, which allowed for

more accurate measurements. Additionally, this sensor was easier to work with and to keep clean.

2.8. Preparative Biotransformations with Anti- and Syn-3-methyl-octane-1,4-diols (1a–b)

A prototype SSF bioreactor was designed and constructed to conduct preparative biotransformations under constant conditions of humidity and oxygenation. During multiple tests of the SSF bioreactor, we confirmed that materials that were used for its design were chosen in an appropriate manner. The cover of the bioreactor (polypropylene), glass capillary, graphite electrodes, probe (Waveshare 9527) and TMP36GT9Z temperature sensor survived multiple sterilization as well as contact with microorganisms and organic solvents. All the elements of the bioreactor were easy to clean and maintain. In the case of damage, electrodes or capillaries can be quickly and simply replaced with new electrodes or capillaries. The air pump that we used in bioreactor ensures good gas exchange.

In preparative biotransformations, bacterial cultures with $OD_{600} = 0.3$ were added to the oilcakes. It was observed that the growth of bacteria was associated with the production of metabolites causing sludge formation and a slight discoloration of the medium. During preliminary studies on preparative scale, it was noticed that the addition of substrate before 4th day after bacterial inoculation led to lower conversion and enantioselectivity of the biotransformation.

On the basis of previous screening experiments for preparative biotransformations of diols (1a–b), the following strains were selected: *Rhodococcus erythropolis* DSM44534, *R. erythropolis* PCM2150 and *Gordonia rubripertincta* PCM2144. Biotransformations of syn-3-methyl-octane-1,4-diol (1b) catalyzed by *R. erythropolis* DSM44534 and *G. rubripertincta* PCM2144 showed 80–100% conversion of the substrate after seven days (Table 6). The isolation yield of a mixture of optically active trans-(+)-(4S,5R) (2a) and cis-(+)-(4R,5R) (2d) whisky lactone isomers in the transformation with *R. erythropolis* DSM44534 was 79.4%. Specific rotations of the enantiomerically enriched trans-(+)-(4S,5R) (2a) ($[\alpha]_D^{20} = +79.7$ (c = 0.25, CH₃OH, ee = 80%, yield = 17.4%); ref. $[\alpha]_D^{20} = +97.0$ (c = 0.34, CH₃OH, ee = 99%) [40]) and cis-(+)-(4R,5R) (2d) ($[\alpha]_D^{20} = +52.1$ (c = 0.2, CH₃OH, ee = 66%, yield = 62%); ref. $[\alpha]_D^{20} = +79.0$ (c = 0.5, CH₃OH, ee = 99%) [40]) whisky lactones were measured and compared with data from the literature. The biotransformation with *G. rubripertincta* PCM2144 was highly selective and afforded only enantiomerically enriched trans-(+)-(4S,5R)-isomer (2a) ($[\alpha]_D^{20} = +61.2$ (c = 0.15, CH₃OH, ee = 66%) with an isolation yield = 68%.

Table 6. Comparison of microbial oxidation of syn-3-methyl-octane-1,4-diol (1b) on linseed cake (in % according to GC).

Strain	Time [days]	Conv. 1b [%]	Products			
			Trans 2a [%]	ee [%]	Cis 2d [%]	ee [%]
<i>Gordonia rubripertincta</i> PCM2144	4	25 (±0.6)	25 (±0.9)	55 (+)-(4S,5R)-2a	0	0
	7	100	100	66 (+)-(4S,5R)-2a	0	0
<i>Rhodococcus erythropolis</i> DSM44534	4	15 (±0.3)	3 (±0.2)	65 (+)-(4S,5R)-2a	12 (±0.5)	54 (+)-(4R,5R)-2d
	7	80 (±1.2)	22 (±0.6)	80(+)-(4S,5R)-2a	58 (±0.5)	66 (+)-(4R,5R)-2d

Analysis of the oxidation of anti-3-methyl-octane-1,4-diol (1a) with *G. rubripertincta* PCM2144 and *R. erythropolis* PCM2150 showed 90–100% conversion of the substrate after seven days (Table 7). In both biotransformations, a mixture of enantiomerically enriched trans-(+)-(4S,5R) (2a) and cis-(−)-(4S,5S) (2c) whisky lactones was obtained. It is worth emphasizing that in the biotransformation of substrate 1a, opposite to the oxidation of 1b, the optically active cis-(−)-(4S,5S) isomer (2c) of whisky lactone was produced. The overall isolation yields of the biotransformations catalyzed by *G. rubripertincta* PCM2144 and *R. erythropolis* PCM2150 were 65% and 61%, respectively. In the transformation with *R. erythropolis* PCM2150, enantiomerically enriched trans-(+)-(4S,5R) (2a) ($[\alpha]_D^{20} = +32.8$ (c = 0.1,

CH₃OH, *ee* = 35%, *yield* = 45%) and *cis*-(-)-(4*S*,5*S*) (**2c**) ($[\alpha]_{\text{D}}^{20} = -54.5$ (*c* = 0.1, CH₃OH, *ee* = 66%, *yield* = 14%); ref. $[\alpha]_{\text{D}}^{20} = -79.0$ (*c* = 0.5, CH₃OH, *ee* = 99%) [40]), whisky lactones were isolated. Preparative oxidation catalyzed by *G. rubripertincta* PCM2144 delivered enantiomerically enriched *trans*-(+)-(4*S*,5*R*)-isomer (**2a**) ($[\alpha]_{\text{D}}^{20} = +60.7$ (*c* = 0.086, CH₃OH, *ee* = 64%, *yield* = 15.1%) and *cis*-(-)-(4*S*,5*S*)-isomer (**2c**) ($[\alpha]_{\text{D}}^{20} = -24.3$ (*c* = 0.1, CH₃OH, *ee* = 25%, *yield* = 42%) of whisky lactones.

Table 7. Comparison of microbial oxidation of *anti*-3-methyl-octane-1,4-diol (**1a**) on linseed cake (in % according to GC).

Strain	Time [days]	Conv. 1a [%]	Products			
			<i>Trans</i> 2a [%]	<i>ee</i> [%]	<i>Cis</i> 2c [%]	<i>ee</i> [%]
<i>Gordonia rubripertincta</i> PCM2144	4	54 (±1.1)	18 (±0.4)	51 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	36 (±0.9)	27 (-)-(4 <i>S</i> ,5 <i>S</i>)- 2c
	7	90 (±1.5)	21 (±0.7)	64 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	69 (±1.1)	25 (-)-(4 <i>S</i> ,5 <i>S</i>)- 2c
<i>Rhodococcus erythropolis</i> PCM2150	4	56 (±0.3)	45 (±0.2)	0	11 (±0.4)	68 (-)-(4 <i>S</i> ,5 <i>S</i>)- 2c
	7	100	77 (±1.3)	35 (+)-(4 <i>S</i> ,5 <i>R</i>)- 2a	23 (±0.8)	66 (-)-(4 <i>S</i> ,5 <i>S</i>)- 2c

3. Materials and Methods

3.1. Microorganisms

Micrococcus luteus PCM525, *Streptomyces griseus* subsp. *griseus* PCM2331, *Dietzia maris* PCM2292, *Rhodococcus coprophilus* PCM2174, *Rhodococcus erythropolis* PCM2150, *Rhodococcus rhodnii* PCM2157, *Rhodococcus rhodochrous* PCM909, *Rhodococcus ruber* PCM2166, *Rhodococcus ruber* PCM2171, *Rhodococcus ruber* PCM2216, *Gordonia bronchialis* PCM2167, *Gordonia rubripertincta* PCM2144 came from the Polish Academy of Sciences. *Dietzia* sp. DSM44016 and *Rhodococcus erythropolis* DSM44534 came from the Department of Chemistry at Wrocław University of Environmental and Life Sciences. Biocatalysts were maintained at 4 °C on PCM agar slants then transferred into conical flasks with PCM medium containing sodium chloride (6 g), glucose (20 g), casein (2 g), bacteriological peptone (10 g) and yeast extract (2 g) dissolved in distilled water (1 L) at 25 °C pH 5.5.

3.2. Materials

A diastereoisomeric mixture of whisky lactones, LiAlH₄ and PCM medium ingredients were purchased from Sigma-Aldrich Chemical Co., St. Louis, MO, USA. Oilseed cakes were purchased from Oleofarm, Wrocław, Poland.

3.3. Measurement of Oilseed Cake Moisture

Five grams of linseed, rapeseed and primrose cakes were weighed into a round-bottom flask. The outlet of the flasks was clogged with cotton wool, and each sample was weighed on an analytical balance and placed in a freeze dryer. The prepared samples were freeze-dried for 24 h and then weighed on an analytical balance. For each oilseed cakes, five repetitions were performed.

3.4. Separation of the *cis/trans*-Whisky Lactones

A diastereoisomeric mixture of *cis/trans*-whisky lactones was separated using column chromatography. For this purpose, a column filled with silica gel dissolved in hexane was prepared, and then, 1.0 g of the *cis/trans*-whisky lactone mixture was applied. The column was eluted with a mixture of hexane:ethylacetate:diethylether:methylene chloride in a ratio of 20:1:1:1. Fractions were collected and controlled using gas chromatography (GC) [8]. Finally, *trans*- (0.430 g) and *cis*- (0.500 g) whisky lactones were collected separately. Spectral data were attached in supporting information (SI).

Trans-whisky lactone ¹H NMR (600 MHz, CDCl₃) δ: 0.91 (t, *J* = 7.2 Hz, 3H, CH₃-4'); 1.13 (d, *J* = 6.5 Hz, 3H, CH₃-4); 1.32–1.42 (m, 3H, CH₂-3', one of CH₂-2'); 1.50 (m, 1H, one of CH₂-2'); 1.60 (m, 1H, one of CH₂-1'); 1.68 (m, 1H, one of CH₂-1'); 2.15–2.25 (m, 2H, one of

CH₂-3, H-4); 2.66 (m, 1H, one of CH₂-3); 4.00 (td, $J = 7.9, 4.0$ Hz, 1H, H-5); ¹³C NMR (150 MHz, CDCl₃): δ 13.89 (C-4'), 17.49 (CH₃-4), 22.49 (C-3'), 27.85 (C-2'), 33.70 (C-1'), 36.08 (C-4), 37.13 (C-3), 87.46 (C-5), 176.61 (C-2); IR (film, cm⁻¹): 1787 (s), 1222 (s), 1187 (s) (Figures S2–S4 SI).

Cis-whisky lactone ¹H NMR (600 MHz, CDCl₃) δ : 0.91 (t, $J = 7.3$ Hz, 3H, CH₃-4'); 1.00 (d, $J = 7.0$ Hz, 3H, CH₃-4); 1.29–1.40 (m, 3H, CH₂-3', one of CH₂-2'); 1.45–1.54 (m, 2H, one of CH₂-2', one of CH₂-1'); 1.65 (m, 1H, one of CH₂-1'); 2.18 (dd, $J = 17.0, 4.0$ Hz, 1H, one of CH₂-3); 2.57 (m, 1H, H-4); 2.67 (dd, $J = 17.0, 7.8$ Hz, 1H, one of CH₂-3); 4.42 (ddd, $J = 10.1, 5.6, 4.1$ Hz, 1H, H-5); ¹³C NMR (150 MHz, CDCl₃): δ 13.82 (CH₃-4), 13.90 (C-4'), 22.51 (C-3'), 28.03 (C-2'), 29.57 (C-4), 33.01 (C-1'), 37.56 (C-3), 83.70 (C-5), 176.94 (C-2); IR (film, cm⁻¹): 1787 (s), 1219 (m), 1180 (s) (Figures S5–S7 SI).

3.5. Chemical Reduction of Whisky Lactones

In the round-bottom flask, 0.420 g of racemic *trans*-whisky lactone (**2a–b**) dissolved in 50 mL of diethyl ether was placed, followed by the addition of 0.128 g LiAlH₄. Similarly, 0.480 g of racemic *cis*-whisky lactone (**2c–d**) was dissolved in diethyl ether, followed by the addition of 0.146 g LiAlH₄. The flask with the attached condenser was placed on a magnetic stirrer. The reduction was carried out for 24 h at 20 °C. The reaction was controlled by thin-layer chromatography (TLC) and gas chromatography (GC). After completion of the reaction, 10% HCl was added to a mixture to spread the excess LiAlH₄. The content of the flask was then transferred to the splitter and extracted three times with diethyl ether. The collected organic layer was then extracted with saturated NaCl solution and dried with MgSO₄, passed through a paper filter and evaporated under reduced pressure. As a result of the reduction, 0.380 g of *anti*-3-methyl-octane-1,4-diol (**1a**) (yield = 90.4%) and 0.456 g of *syn*-3-methyl-octane-1,4-diol (**1b**) (yield = 95%) were obtained. Spectral data were attached in supporting information (SI).

Anti-3-methyl-octane-1,4-diol (**1a**) ¹H NMR (600 MHz, CDCl₃) δ : 0.87 (d, $J = 6.8$ Hz, 3H, CH₃-3); 0.89 (t, $J = 7.1$ Hz, 3H, CH₃-8); 1.22–1.36 (m, 3H, one of CH₂-6, CH₂-7); 1.38–1.46 (m, 3H, CH₂-5, one of CH₂-6); 1.50 (m, 1H, one of CH₂-2); 1.67–1.77 (m, 2H, one of CH₂-2, H-3); 2.81 i 3.00 (two s, 2H, 2xOH); 3.55 (m, 1H, H-4); 3.62 (ddd, $J = 10.9, 7.1, 5.0$ Hz, 1H, one of CH₂-1); 3.73 (ddd, $J = 10.9, 6.4, 5.0$ Hz, 1H, one of CH₂-1); ¹³C NMR (150 MHz, CDCl₃) δ : 13.89 (CH₃-3), 14.12 (C-8), 22.79 (C-7), 28.70 (C-6), 33.35 (C-5), 35.99 (C-3), 36.20 (C-2), 60.65 (C-1), 74.97 (C-4); IR (film, cm⁻¹): 3342 (s), 1475 (m), 1395 (m), 1065 (m), 1018 (m) (Figures S8–S10 SI).

Syn-3-methyl-octane-1,4-diol (**1b**) ¹H NMR (600 MHz, CDCl₃) δ : 0.89 (t, $J = 7.1$ Hz, 3H, CH₃-8); 0.92 (d, $J = 6.8$ Hz, 3H, CH₃-3); 1.23–1.36 (m, 3H, one of CH₂-6, CH₂-7); 1.37–1.51 (m, 3H, CH₂-5, one of CH₂-6); 1.56 (m, 1H, one of CH₂-2); 1.62–1.70 (m, 2H, one of CH₂-2, H-3); 3.13 (s, 2H, 2xOH); 3.38 (ddd, $J = 8.4, 5.5, 3.3$ Hz, 1H, H-4); 3.59 (ddd, $J = 11.4, 6.9, 5.1$ Hz, 1H, one of CH₂-1); 3.72 (ddd, $J = 11.4, 6.7, 5.0$ Hz, 1H, one of CH₂-1); ¹³C NMR (150 MHz, CDCl₃) δ : 14.12 (C-8), 16.60 (CH₃-3), 22.81 (C-7), 28.06 (C-6), 34.14 (C-5), 35.26 (C-2), 36.43 (C-3), 60.31 (C-1), 75.82 (C-4); IR (film, cm⁻¹): 3333 (s), 1480 (s), 1386 (s), 1069 (s), 1018 (s) (Figures S11–S13 SI).

3.6. Screening Scale Biotransformations

Five grams of oilseed cake (linseed, rapeseed or primrose) was weighed into 100 mL tapered flasks and then sterilized at 121 °C at a pressure of 1 atm. The medium was inoculated with 0.5 mL of preprepared cultures of bacteria at OD₆₀₀ = 0.3. The prepared bacterial cultures were placed for 4 days at 30 °C. After this time, 0.01 g of the substrate (*anti*-3-methyl-octane-1,4-diol (**1a**) or *syn*-3-methyl-octane-1,4-diol (**1b**)) dissolved in 0.5 mL of acetone and 0.5 mL of water was sprayed onto each of the flasks. For simple extraction, ethyl acetate (25 mL) was added to the samples and shaken for 5 min at 200 rpm in Falcon tubes. The organic phase was transferred to a vial and dehydrated by anhydrous MgSO₄. Then, it was filtered through a paper filter to a GC vial. Biotransformation was controlled after three and seven days on the GC. Control experiments were also performed in which

microorganisms were cultured on the medium without the addition of substrate to check their metabolites. The stability of the substrate was also checked by the addition of the substrate to the medium without microorganisms.

3.7. Statistical Analysis

All the described experiments were performed in triplicate, and the values presented in the tables are the mean of the obtained results. Student's *t*-test showed that all the data did not differ significantly. Additionally, the values of the standard deviation were calculated for the conversion and a percentage of whisky lactone isomers are shown in the tables. Statistical analyses were performed by Past 4.02.

3.8. Preparative Biotransformations

Linseed cake (50 g) was placed in the bioreactor vessel. The bioreactor was sterilized at 121 °C for 15 min. Subsequently, the medium was inoculated with 5 mL of preprepared cultures of bacteria at $OD_{600} = 0.3$. Then, 75 mL of sterile water was added to obtain 60% medium moisture. The bioreactor was connected to the rest of the apparatus and placed in a thermostatic cabinet at 30 °C. The culture was incubated for 4 days with continuous gas exchange under 60% humidity. The air pump, guaranteed gas exchange and sterile flow of air in the chamber, was turned on for 5 min with 30 min intervals. Then, 0.1 g of the substrate (*anti*-3-methyl-octane-1,4-diol (**1a**) or *syn*-3-methyl-octane-1,4-diol (**1b**)) dissolved in 2.5 mL of acetone and 2.5 mL of water was sprayed onto the culture. Samples were extracted after 2, 3, 4, 5 and seven days and checked by GC to estimate the progress of the biotransformation.

3.9. Design of the Bioreactor

The SSF bioreactor is based on calculating the voltage measurement. A Raspberry Pi Zero W was connected to a 10-bit analog-to-digital converter MCP3008 and to an opto-isolated two-channel relay. For both relays, as a control signal, 3.3 V signal from a GPIO pins was used. Relays were used to control two 4.8 W air pumps with maximal efficiency of 2 × 270 L/h and continuous regulation. Two graphite electrodes, sensor with a probe for measuring soil moisture Waveshare 9527, steel tube for temperature sensor, one glass capillary with tapered tip responsible for water additions and two glass capillary with microbiological filters with 0.2 µm pore size for gas exchange were placed in the bioreactor chamber. To minimize the impact of electric current, the humidity sensor electrodes were connected to the MOSFET transistor as a switch. Microbiological filters with 0.2 µm input and output as well as microbiological filters with input of air in bottles with sterile water guaranteed sterile air conditions. To connected air wires Teflon hoses were used. The chamber was made of polypropylene to be sterilized. The dimensions of the vessel were 120 mm × 85 mm × 65 mm and were selected to be able to work in the chamber under sterile conditions. The graphite electrodes connected to MCP3008 were 22 mm in diameter and 160 mm in height (Figures S14–S17 SI).

3.10. Extraction Methods

3.10.1. Simple Extraction

When the biotransformation was completed, growth medium with microorganisms was transferred into a 1000 mL flask and extracted three times with ethyl acetate (100 mL). During the first extraction, the reaction mixture was shaken with solvent for 4 h, and then the organic phase was filtered under reduced pressure. Subsequent extractions were carried out by analogy lasting 2 h and 1 h, respectively. The collected organic phase was transferred into a flask and dehydrated by anhydrous $MgSO_4$. Then, it was filtered through a paper filter, and the solvent was evaporated under reduced pressure.

3.10.2. Steam Distillation

After biotransformation, growth medium with biomass was transferred into a 500 mL round-bottom flask, and then 200 mL of distilled water was added. The flask was connected to the distillation apparatus. Distillation was carried out for 2 h, and the distillate was collected into a 250 mL round-bottom flask. The distillates were transferred to a 500 mL splitter and then extracted three times with 80 mL of ethyl acetate. Then, the extract was dried with anhydrous MgSO_4 , and the organic solvent was evaporated under reduced pressure.

3.10.3. Steam Distillation from the Extract

In this modification of steam distillation, the content of the bioreactor vessel was transferred into a 1000 mL flask and extracted three times with ethyl acetate (100 mL). During the first extraction, the reaction mixture was shaken with solvent for 4 h, and then the organic phase was filtered under reduced pressure. Subsequent extractions were carried out by analogy lasting 2 h and 1 h. The organic phase was transferred to a flask and dehydrated by anhydrous MgSO_4 . Then, it was filtered through a paper filter, and the solvent was evaporated under reduced pressure. The extract obtained from three extractions was placed in a 250 mL round-bottom flask, and 100 mL of distilled water was added and connected to the distillation apparatus. Distillation was carried out for 2 h, and 250 mL of distillate was collected. The distillates were transferred to a 500 mL splitter and then extracted three times with 80 mL of ethyl acetate. Then, the extract was dried with anhydrous MgSO_4 , and the organic solvent was evaporated under reduced pressure.

3.10.4. Extraction with a Drying Apparatus

After biotransformation, reaction mixture was placed in a 500 mL round-bottom flask, and then 200 mL of distilled water was added. The sample flask was heated for 2 h. The vapors were condensed by means of a cold refrigerant. After extraction, 1 mL of cyclohexane containing biotransformation products was collected.

3.11. Analysis Procedure

Separation of the diastereoisomeric mixture of *cis/trans*-whisky lactones and chemical reduction of whisky lactones to corresponding diols were controlled by thin layer chromatography (TLC), using aluminum foil plates coated with silica gel. Compounds were detected by spraying the plates with 1% $\text{Ce}(\text{SO}_4)_2$ and 2% $\text{H}_3[\text{P}(\text{Mo}_3\text{O}_{10})_4]$ in 10% H_2SO_4 . Gas chromatography analysis (GC, FID, carrier gas H_2) was carried out on Agilent Technologies 7890N (GC System, Santa Clara, CA, USA). Enantiomeric excesses of the products were determined on chiral column Cyclosil-B (30 m \times 0.25 mm \times 0.25 μm , Santa Clara, CA, USA) according to the next temperature program: 80 $^\circ\text{C}$, 160 $^\circ\text{C}$ (3 $^\circ\text{C}/\text{min}$), 250 $^\circ\text{C}$ (20 $^\circ\text{C}/\text{min}$) (3 min). Samples (2 μL) were injected with split 9:1; the flow of carrying gas was 1 mL/min. The total run time was 34.0 min. Retention times were established as follow: t_R = 20.74 min for *trans*-(+)-(4*S*,5*R*) (**2a**), t_R = 21.05 min for *trans*-(-)-(4*R*,5*S*) (**2b**), t_R = 22.42 min for *cis*-(-)-(4*S*,5*S*) (**2c**), t_R = 22.54 min for *cis*-(+)-(4*R*,5*R*) (**2d**) (Figure S1 SI). The substrates were determined on the chiral column CP-Chirasil L-Val (25 m \times 0.25 mm \times 0.12 μm , Santa Clara, CA, USA) according to the next temperature program: 80 $^\circ\text{C}$, 165 $^\circ\text{C}$ (3 $^\circ\text{C}/\text{min}$), 200 $^\circ\text{C}$ (20 $^\circ\text{C}/\text{min}$) (1 min). Samples (2 μL) were injected with split 9:1; the flow of carrying gas was 1 mL/min. The total run time was 31.0 min. Retention times were established as follow: t_R = 18.553 for *anti*-3-methyl-octane-1,4-diol (**1a**), t_R = 18.630 min for *syn*-3-methyl-octane-1,4-diol (**1b**). The structures of the compounds were confirmed on the basis of ^1H NMR and ^{13}C NMR, which were recorded for CDCl_3 solutions on a Bruker Avance DRX 600 (600 MHz) spectrometer (Billerica, MA, USA). IR spectra were determined using FTIR Thermo-Mattson IR 300 Spectrometer. Optical rotations were measured on a Jasco P-2000 Polarimeter.

3.12. Moisture Analysis

To ensure maximum chemical protection and to minimize the impact of metals on microorganisms, we decided to use graphite electrodes to measure the moisture content. A sensor with a probe for measuring soil moisture Waveshare 9527 was also checked to compare them with graphite electrodes and determine which electrodes would be better for controlling the humidity. The moisture sensor was calibrated using an oil cake with known water content. The bioreactor chamber was placed in a temperature controller at an air temperature of 30 °C. The preliminary study showed that an equilibrium state was established in 15 min, so the measurements were carried out after this time. Water was added to the bioreactor vessel in a liquid state by spraying through a septum with small holes ($d = 1$ mm). The moisture sensor monitored the oil cake moisture and water was added automatically when the humidity level in the bioreactor vessel decreased by 3% from the set point. Sensor measurements were fluctuating in range $\pm 1\%$.

The relation between the water additive and voltage was determined using an analog digital converter. A separate curve depending on the moisture from the analog to digital converter was designed for each of the oilcakes. We also examine the effect of temperature and electrode distance on the response of the sensor. When we increased the moisture, the resistance between electrodes decreased, which resulted in more voltage. Electrodes were placed at a distance of 3 cm from each other and submerged in the culture to a depth of 1 cm. Measurement of the water content depending on the conductivity was carried out by adding water successively, which corresponds to 5% to 90% by increasing the oilcake content by 5 to 50 g. Research was carried out at a temperature of 30 °C. Moisture was calculated by the following Equation (1) which allows to calculate the amount of water required to achieve the desired humidity value taking into consideration the water contained presently in the cake.

$$Ww = Ow * \frac{W}{100\% - W} - Ow * Mw \quad (1)$$

Ww —Water weight [g]

Ow —Oilcake weight [g]

W —Moisture content that we need to obtain [%]

Mw —Moisture content in oilcakes [%]

The amount of water added ranged from 2.6 g for 5% moisture to 450 g for 90% moisture. Each addition of water required 15 min of waiting for water absorption. Then, measurements were carried out every 10 s until 10 matching results were obtained.

3.13. Software

In the presented bioreactor, we used a Raspbian Stretch Linux distribution specifically designed for Raspberry Pi. To control the relay and MCP3008, Python programming was used. When the moisture value was too low, the resistance between the graphite electrodes increased, and one of the GPIO pins that controlled the relay changed the LOW/HIGH state. The air pumps, enabled by the relay, pumped sterile air to the bottle with water, the pressure pushed water to capillary, and consequently, the medium was moistened. The second pump was used to exchange the gas by removal of carbon dioxide and other gases and was controlled by the GPIO pins switched on at certain intervals. The temperature sensor TMP36GT9Z was used only for remote temperature reading and was not connected to the heating cabinet. All data from sensors were collected and sent to a server.

4. Conclusions

The byproducts from the oil industry can be used as a valuable microbial medium for the bacterial oxidation process, leading from diols to whisky lactones. Biotransformations carried out on a preparative scale delivered corresponding enantiomerically enriched isomers *trans*-(+)-(4*S*,5*R*) (**2a**), *cis*-(-)-(4*S*,5*S*) (**2c**) and *cis*-(+)-(4*R*,5*R*) (**2d**). Among the different

oil cakes tested, biotransformations carried out on linseed cake were characterized by a high conversion and stereoselectivity. During the research, it was confirmed that biotransformations should be carried out separately for *anti*- (1a) and *syn*-3-methyl-octane-1,4-diol (1b) because the oxidation of the diastereoisomeric mixture of diols was characterized by low stereoselectivity. Steam distillation followed by simple extraction with the use of organic solvents was proven to be the most efficient method of extracting products after biotransformation. This method allowed for the extraction of whisky lactones without the fats in the oilcake. In our opinion, our bioreactor prototype is definitively a better alternative to classic biotransformations performed in Erlenmeyer flasks. The measurements of moisture in the solid medium using graphite electrodes were satisfactory, and the irrigation and gas exchange system worked properly. Our results showed that by using simple solutions, it is possible to create a fully functional bioreactor prototype.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2073-4344/11/3/320/s1>, Figure S1: Chromatogram of the mixture of *trans* and *cis* whisky lactone, Figure S2: ¹H NMR spectrum of *trans*-whisky lactone, Figure S3: ¹³C NMR spectrum of *trans*-whisky lactone, Figure S4: IR spectrum of *trans*-whisky lactone, Figure S5: ¹H NMR spectrum of *cis*-whisky lactone, Figure S6: ¹³C NMR spectrum of *cis*-whisky lactone, Figure S7: IR spectrum of *cis*-whisky lactone, Figure S8: ¹H NMR spectrum of *anti*-3-methyl-octane-1,4-diol, Figure S9: ¹³C NMR spectrum of *anti*-3-methyl-octane-1,4-diol, Figure S10: IR spectrum of *anti*-3-methyl-octane-1,4-diol, Figure S11: ¹H NMR spectrum *syn*-3-methyl-octane-1,4-diol, Figure S12: ¹³C NMR spectrum *syn*-3-methyl-octane-1,4-diol, Figure S13: IR spectrum *syn*-3-methyl-octane-1,4-diol, Figure S14: A bioreactor vessel with graphite electrodes, a vessel with water, air and water pumps, Figure S15: Control system in the plastic cover, Figure S16: Moisture sensor, Figure S17: Bacteria in the linseed cake.

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References

1. Pandey, A. Solid-state fermentation. *Biochem. Eng. J.* **2003**, *13*, 81–84. [[CrossRef](#)]
2. Kapilan, R. Solid state fermentation for microbial products: A review. *Appl. Sci. Res.* **2015**, *7*, 21–25.
3. Pandey, A.; Soccol, C.R.; Mitchell, D. New developments in solid state fermentation: I-bioprocesses and products. *Process. Biochem.* **2000**, *35*, 1153–1169. [[CrossRef](#)]
4. Singhanian, R.R.; Patel, A.K.; Soccol, C.R.; Pandey, A. Recent advances in solid-state fermentation. *Biochem. Eng. J.* **2009**, *44*, 13–18. [[CrossRef](#)]
5. Rodri, S. Application of solid-state fermentation to food industry—A review. *J. Food Eng.* **2006**, *76*, 291–302.
6. Ramachandran, S.; Singh, S.K.; Larroche, C.; Soccol, C.R.; Pandey, A. Oil cakes and their biotechnological applications—A review. *Bioresour. Technol.* **2007**, *98*, 2000–2009. [[CrossRef](#)]
7. Soccol, C.R.; da Costa, E.S.F.; Letti, L.A.J.; Karp, S.G.; Wojciechowski, A.L.; de Souza Vandenberghe, L.P. Recent developments and innovations in solid state fermentation. *Biotechnol. Res. Innov.* **2017**, *1*, 52–71. [[CrossRef](#)]
8. Kannan, T.R.; Kanagaraj, C. Molecular characteristic of α -amylase enzymes producing from *Bacillus licheniformis* (JQ946317) using solid state fermentation. *Biocatal. Agric. Biotechnol.* **2019**, *20*, 1–6. [[CrossRef](#)]

9. Liguori, R.; Amore, A.; Faraco, V. Waste valorization by biotechnological conversion into added value products. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 6129–6147. [[CrossRef](#)] [[PubMed](#)]
10. Papagianni, M. Recent Advances in Solid-State Fermentation Applications for the Food Industry. *Curr. Biochem. Eng.* **2013**, *1*, 2–8. [[CrossRef](#)]
11. Rodríguez Madrera, R.; Pando Bedriñana, R.; Suárez Valles, B. Production and characterization of aroma compounds from apple pomace by solid-state fermentation with selected yeasts. *LWT Food Sci. Technol.* **2015**, *64*, 1342–1353. [[CrossRef](#)]
12. Raghavarao, K.S.M.S.; Ranganathan, T.V.; Karanth, N.G. Some engineering aspects of solid-state fermentation. *Biochem. Eng. J.* **2003**, *13*, 127–135. [[CrossRef](#)]
13. Castilho, L.R.; Polato, C.M.S.; Baruque, E.A.; Sant’Anna, G.L.; Freire, D.M.G. Economic analysis of lipase production by *Penicillium restrictum* in solid-state and submerged fermentations. *Biochem. Eng. J.* **2000**, *4*, 239–247. [[CrossRef](#)]
14. Boratyński, F.; Szczepańska, E.; Grudniewska, A.; Skalny, B.; Olejniczak, T. A novel approach for microbial synthesis of enantiomerically pure whisky lactones based on solid-state fermentation. *Molecules* **2018**, *23*, 659. [[CrossRef](#)]
15. Chreptowicz, K.; Wielechowska, M.; Główczyk-Zubek, J.; Rybak, E.; Mierzejewska, J. Production of natural 2-phenylethanol: From biotransformation to purified product. *Food Bioprod. Process.* **2016**, *100*, 275–281. [[CrossRef](#)]
16. Nagy, V.; Toke, E.R.; Keong, L.C.; Szatzker, G.; Ibrahim, D.; Omar, I.C.; Szakács, G.; Poppe, L. Kinetic resolutions with novel, highly enantioselective fungal lipases produced by solid state fermentation. *J. Mol. Catal. B Enzym.* **2006**, *39*, 141–148. [[CrossRef](#)]
17. Marie, L.; Gori, K.; Agerlin, M.; Jespersen, L.; Arneborg, N. Flavour compound production by *Yarrowia lipolytica*, *Saccharomyces cerevisiae* and *Debaryomyces hansenii* in a cheese-surface model. *Int. Dairy J.* **2011**, *21*, 970–978.
18. Marco, B.A.D.; Rechelo, B.S.; Tótolí, E.G.; Kogawa, A.C.; Regina, H.; Salgado, N. Evolution of green chemistry and its multidimensional impacts: A review. *Saudi Pharm. J.* **2019**, *27*, 1–8. [[CrossRef](#)]
19. Braga, A.; Belo, I. Biotechnological production of gamma-decalactone, a peach like aroma, by *Yarrowia lipolytica*. *World J. Microbiol. Biotechnol.* **2016**, *32*, 169. [[CrossRef](#)]
20. Ashok, A.; Doriya, K.; Rao, D.R.M.; Kumar, D.S. Design of solid state bioreactor for industrial applications: An overview to conventional bioreactors. *Biocatal. Agric. Biotechnol.* **2017**, *9*, 11–18. [[CrossRef](#)]
21. Rayhane, H.; Josiane, M.; Gregoria, M.; Yiannis, K.; Nathalie, D.; Ahmed, M.; Sevastianos, R. From flasks to single used bioreactor: Scale-up of solid state fermentation process for metabolites and conidia production by *Trichoderma asperellum*. *J. Environ. Manage.* **2019**, *252*, 109496. [[CrossRef](#)]
22. Maga, J.A. Oak lactones in alcoholic beverages. *Food Rev. Int.* **1996**, *12*, 105–130. [[CrossRef](#)]
23. Günther, C.; Mosandl, A. Stereoisomere Aromastoffe, XII. 3-Methyl-4-octanolid—“Quercuslacton, Whiskylacton”—Struktur und Eigenschaften der Stereoisomeren. *Liebigs Ann. der Chemie* **1986**, *1986*, 2112–2122. [[CrossRef](#)]
24. Abbott, N.; Puech, J.L.; Bayonove, C.; Baumes, R. Determination of the Aroma Threshold of the *cis* and *trans* Racemic Forms of β -Methyl- γ -Octalactone by Gas Chromatography-Sniffing Analysis. *Am. J. Enol. Vitic.* **1995**, *46*, 292–294.
25. Suzukt, Y.; Mori, W.; Ishizone, H.; Naito, K.; Honda, T. Concise enantiospecific syntheses of (+)-Eldanolide and (–)-*cis*-whisky lactone. *Tetrahedron Lett.* **1992**, *33*, 4931–4932. [[CrossRef](#)]
26. Ito, K.; Yoshitake, M.; Katsuki, T. Chiral bipyridine and biquinoline ligands: Their asymmetric synthesis and application to the synthesis of *trans*-whisky lactone. *Tetrahedron* **1996**, *52*, 3905–3920. [[CrossRef](#)]
27. Jiang, X.; Fu, C.; Ma, S. A concise synthesis of (–)- and (+)-*trans*-whisky lactones. *Eur. J. Org. Chem.* **2010**, *2010*, 687–693. [[CrossRef](#)]
28. Pisani, L.; Superchi, S.; D’Elia, A.; Scafato, P.; Rosini, C. Synthetic approach toward *cis*-disubstituted γ - and δ -lactones through enantioselective dialkylzinc addition to aldehydes: Application to the synthesis of optically active flavors and fragrances. *Tetrahedron* **2012**, *68*, 5779–5784. [[CrossRef](#)]
29. Armstrong, A.; Ashraff, C.; Chung, H.; Murtagh, L. Oxidative rearrangement of 2-alkoxy-3,4-dihydro-2H-pyrans: Stereocontrolled synthesis of 4,5-*cis*-disubstituted tetrahydrofuranones including whisky and cognac lactones and crobarbatic acid. *Tetrahedron* **2009**, *65*, 4490–4504. [[CrossRef](#)]
30. Xie, H.; Lu, J.; Gul, Y.; Gao, L.; Song, Z. (HMe₂SiCH₂)₂: A useful reagent for B(C₆F₅)₃-catalyzed reduction–lactonization of keto acids: Concise syntheses of (–)-*cis*-whisky and (–)-*cis*-cognac lactones. *Synlett* **2017**, *28*, 2453–2459.
31. Boratyński, F.; Smuga, M.; Wawrzeńczyk, C. Lactones 42. Stereoselective enzymatic/microbial synthesis of optically active isomers of whisky lactone. *Food Chem.* **2013**, *141*, 419–427. [[CrossRef](#)]
32. Boratyński, F.; Szczepańska, E.; Grudniewska, A.; Olejniczak, T. Microbial kinetic resolution of aroma compounds using solid-state fermentation. *Catalysts* **2018**, *8*, 28. [[CrossRef](#)]
33. Boratyński, F.; Danciewicz, K.; Paprocka, M.; Gabryś, B.; Wawrzeńczyk, C. Chemo-enzymatic synthesis of optically active γ - and δ -decalactones and their effect on aphid probing, feeding and settling behavior. *PLoS ONE* **2016**, *11*, e0146160. [[CrossRef](#)]
34. Boratyński, F.; Szczepańska, E.; De Simeis, D.; Serra, S.; Brenna, E. Bacterial biotransformation of oleic acid: New findings on the formation of γ -dodecalactone and 10-ketostearic acid in the culture of *Micrococcus luteus*. *Molecules* **2020**, *25*, 3024. [[CrossRef](#)]
35. Boratyński, F.; Pannek, J.; Walczak, P.; Janik-Polanowicz, A.; Huszcza, E.; Szczepańska, E.; Martin-Rojas, E.; Olejniczak, T. Microbial alcohol dehydrogenase screening for enantiopure lactone synthesis: Down-stream process from microtiter plate to bench bioreactor. *Process. Biochem.* **2014**, *49*, 1637–1646. [[CrossRef](#)]
36. Graminha, E.B.N.; Gonçalves, A.Z.L.; Pirota, R.D.P.B.; Balsalobre, M.A.A.; Da Silva, R.; Gomes, E. Enzyme production by solid-state fermentation: Application to animal nutrition. *Anim. Feed Sci. Technol.* **2008**, *144*, 1–22. [[CrossRef](#)]

37. Gutierrez, C.; Rubilar, M.; Jara, C.; Verdugo, M.; Sineiro, J.; Shene, C. Flaxseed and Flaxseed cake as a source of compounds for food industry. *J. Soil Sci. Plant. Nutr.* **2009**, *10*, 454–463. [[CrossRef](#)]
38. Stasiniewicz, T.; Niwińska, B.; Strzetelski, J.; Kowalczyk, J.; Maciaszek, K.; Bilik, K. Nutritive value of evening primrose (*Oenothera paradoxa*) cake for ruminants. *J. Anim. Feed Sci.* **1998**, *7*, 187–195. [[CrossRef](#)]
39. Liu, J.; Du, C.; Beaman, H.; Monroe, M. Characterization of phenolic acid antimicrobial and antioxidant structure-property relationships. *Pharmaceutics* **2020**, *21*, 419. [[CrossRef](#)]
40. Wilkinson, K.L.; Elsey, G.M.; Prager, R.H.; Tanaka, T.; Sefon, M.A. Precursors to oak lactone. Part 2: Synthesis, separation and cleavage of several β -D-glucopyranosides of 3-methyl-4-hydroxyoctanoic acid. *Tetrahedron* **2004**, *60*, 6091–6100. [[CrossRef](#)]