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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
07 May 2020 (07.05.2020)

WIPO | PCT

(10) International Publication Number
WO 2020/089934 A1

(51) International Patent Classification:
C07C 215/08 (2006.01) *C12P 7/02* (2006.01)
C07C 209/68 (2006.01) *C12P 41/00* (2006.01)

(21) International Application Number:
PCT/IN2019/050797

(22) International Filing Date:
30 October 2019 (30.10.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
201821041686 02 November 2018 (02.11.2018) IN

(72) Inventor; and

(71) Applicant: **YADAV, Ganapati Dadasaheb** [IN/IN]; 7/5/6 Hill Crest Society, Bhavani Nagar, Near Vijay Nagar, Marol Maroshi Road, Andheri East, Mumbai 400 059, Maharashtra (IN).

(72) Inventors: **MOHIRE, Shalaka Sanjiv**; Chemical Engineering Department, Institute of Chemical Technology, Nathalal Parekh Marg, Matunga (east), mumbai 400 019, Maharashtra (IN). **ASHILY, Rajendran**; Chemical Engineering Department, Institute of Chemical Technology, Nathalal Parekh Marg, Matunga (east), mumbai 400 019, Maharashtra (IN).

(74) Agent: **DHAKE, Poonam**; 7/5/6 Hill Crest Society, Bhavani Nagar, Near Vijay Nagar, Marol Maroshi Road, Andheri East, Mumbai 400 059, Maharashtra (IN).

(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).

Declarations under Rule 4.17:
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

WO 2020/089934 A1

(54) Title: BIOCATALYTICAL PROCESS FOR RACEMIZATION OF D-EPHEDRINE

(57) Abstract: This invention relates to methods for racemization of dextro-rotatory ephedrine via enzymatic kinetic conversion, and is particularly useful for conversion of dextro- rotatory ephedrine (d- ephedrine) to racemic mixture of dextro-rotatory ephedrine (d-ephedrine) and levo-rotatory ephedrine (l-ephedrine) to recover the levo-rotatory ephedrine that exhibit potential bronchodilatory and anti-hypotensive activities. The process provides a suspension of *Rhizopus Oryzae* fungi pellets in diammonium phosphate buffer having pH in the range of pH 5 to 9 and effective sonication to extract specific enzymes for inversion of functional groups present on the chiral carbon atom of d-ephedrine molecule at low temperature which has advantages of working at lower temperature range (20 to 50 °C), lower energy consumption, lesser formation of by-products.

TITLE OF THE INVENTION:

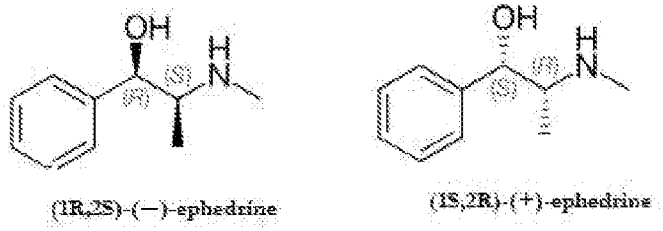
BIOCATALYTICAL PROCESS FOR RACEMIZATION OF D-EPHEDRINE

FIELD OF THE INVENTION

The present invention relates to a process for racemization of dextro-rotatory ephedrine via biocatalytic transformation and is particularly useful for conversion of dextro- rotatory ephedrine (d- ephedrine) to racemic mixture of dextro-rotatory ephedrine (d-ephedrine) and levo-rotatory ephedrine (l-ephedrine). The present invention provides an effective process to recover the levo-rotatory ephedrine that exhibit potential bronchodilatory and anti-hypotensive activities.

BACKGROUND AND PRIOR ART OF THE INVENTION

The synthetic production of racemic ephedrine results into formation of detro-rotatory ephedrine (+ephedrine) along with the levo-rotatory ephedrine (-ephedrine), of which the latter one, l-form is comparatively a more active compound. The d-form cannot be utilized in pharmaceutical companies and thus remains as an undesirable compound.



(1) (2)

SCHEME 1: Structure of (1) levo-rotatory ephedrine, (2) dextro-rotatory ephedrine.

The IUPAC name of the enantiomeric compounds 1 and 2 are (1R,2S)-2-methylamino-1-phenylpropan-1-ol and (1S,2R)-2-methylamino-1-phenylpropan-1-ol, respectively.

Racemization is the process when the enantiomer is converted into racemic modification (i.e. chemical reactions) or when one pure form of an enantiomer is converted into equal proportion of both enantiomers (levo- and dextro-), forming a racemate.

There are two main strategies for the preparation of enantiopure compounds. The first is known as chiral resolution. This method involves preparing the compound in racemic form, and separating it into its isomers.

The second strategy is asymmetric synthesis: the use of various techniques to prepare the desired compound in high enantiomeric excess. Techniques encompassed include the use of chiral starting materials (chiral pool synthesis), the use of chiral auxiliaries and chiral catalysts, and the application of asymmetric induction.

As racemic ephedrine is mixture of dextro-rotatory ephedrine (+ephedrine) along with the levo-rotatory ephedrine (-ephedrine), of which the latter one, levo-form is comparatively a more active compound. The kinetic resolution techniques like crystallization and enzymatic resolution are well established techniques for chiral separation of desired enantiomer from racemic mixture. After such resolution, the dextro-form cannot be utilized in pharmaceutical companies and thus remains as an undesirable compound.

Therefore, need is to synthesize levo-rotatory ephedrine (Scheme 1: compound 1) as a pure and active enantiomer by converting previously separated dextro-rotatory ephedrine (Scheme 1: compound 2) in to levo-rotatory ephedrine through racemization.

Earlier racemization of d-ephedrine to dl-ephedrine was reported with alkali metal alcoholates. This process possesses disadvantages such as, formation of unwanted materials due to side reactions, poor yield and extensive decomposition of the reaction mixture. This process has tendency to continuously liberated water during the reaction, which accelerates decomposition (Georg et al., 1939).

Another method for the racemization of d-ephedrine reported uses the amides and hydrides of alkali and alkaline earth metals. The amount of amide or hydride used was small and ammonia or hydrogen gas was liberated during the process, which tends to prevent decomposition (Tabern and Donalee, 1940).

With kinetic resolution each enantiomer can be obtained with a maximum yield of only 50% from its racemate. Whereas, a kinetic resolution with simultaneously racemization of the remaining unreacted enantiomer can be done, in a process called dynamic kinetic resolution (DKR). With this process a single enantiomer with maximum theoretical yield of 100% from its racemate can be obtained. The conditions required for both racemization and kinetic resolution, such as pH, temperature, must be compatible. Most important issue is catalyst must not interact with the product of the reaction.

Hence, existing methods mainly comprise of chemical processes which followed the harsh reaction conditions, while few of them were consistent with mild conditions, usually using the transition metal catalysts. Racemization of enantiomeric ephedrine was carried on by treating it with higher temperature above the normal with an alkali alcoholate. Similarly, 1-aryl-aklylamines were subjected to racemization by using metal alkoxides in dimethyl sulphoxides optionally in the presence of appropriate alcohols. However, the known racemization processes, are not suitable for industrial scale due to unsatisfactory yield or insufficient quality of products or industrially disadvantageous catalysts or some working up problems. Hence, this approach is made towards biocatalysis with lower temperature range (0-110 °C), lower energy consumption, lesser formation of by-products, non-toxic and safer process when used correctly, reusable, biodegradable, stereo- and regio-selective unlimited production capabilities, industrially scalable, economic process and having good yield in terms of racemic compound which is on demand.

Accordingly, efforts are made to use biocatalytic racemization by selecting some particular strain of microorganisms, secreting enzymes that cause the inversion of functional groups so as to form the racemic mixtures. Different strains act

differently for varied number of substrates. Hence screening of particular strain, extraction and purification of enzymes are few of the challenges faced. But once a particular strain showing positive results is obtained, it is advantageous over chemical methods due to its safer, milder process conditions.

Hence, the inventors of the present invention developed an improved method for converting a dextro-rotatory ephedrine to racemate of ephedrine (Both levo and dextro form in mixture), further separating levo- rotatory ephedrine from the reaction mass as active compound and reusing the separated out dextro-rotatory ephedrine for same racemization reaction process. The present invention provides a biocatalytic process for racemization of dextro-rotatory ephedrine, wherein whole cell enzyme biocatalysis has been used which has an add-on advantage in the racemization process. By using the whole cells, the cost of process of extraction, purification, separation and maintenance of specific enzymes can be minimized.

Hence, the present invention is a solution for converting the undesired d-ephedrine produced during synthesis of ephedrine into a racemic mixture to again recover the desirable l-form of ephedrine from it. Thus, the d-ephedrine is made into a reusable form.

OBJECT OF THE INVENTION

The main objective of present invention is to provide a process for racemization of dextro-rotatory ephedrine via biocatalytic transformation.

One more objective of present invention is to provide a process of conversion of dextro- rotatory ephedrine (d- ephedrine) to racemic mixture of dextro-rotatory ephedrine (d-ephedrine) and levo-rotatory ephedrine (l-ephedrine).

Yet one of the objective of present invention is to provide a process for racemization of dextro-rotatory ephedrine having lower temperature range (20 to 50 °C), lower energy consumption, lesser formation of by-products, non-toxic and safer industrial viable process.

Another objective of present invention is to provide a process for synthesis of pure racemic d and l-ephedrine without formation of other undesired by-products like pseudo-ephedrine.

SUMMARY OF THE INVENTION

The present invention relates to a process for racemization of dextro-rotatory ephedrine via biocatalytic transformation and is particularly useful for conversion of dextro- rotatory ephedrine (d- ephedrine) to racemic mixture of dextro-rotatory ephedrine (d-ephedrine) and levo-rotatory ephedrine (l-ephedrine).

In accordance to embodiment, the present invention provides a process for Racemization of dextro- rotatory ephedrine, the process comprising of:

Step 1: Preparation of suspension of *Rhizopus Oryzae* fungi pellets in diammonium phosphate buffer having pH in the range of pH 5 to 9

Step 2: Ultrasonicating the step 1 suspension for time between 3 to 10 min,

Step 3: Addition of d-ephedrine in ultrasonicated suspension of step 2) and carrying out reaction at temperature between 25 to 35 °C for 10 to 36 h under constant stirring,

Step 4: Separation of liquid from solid mass by centrifugation at 300 to 10000 rpm.

Wherein concentration of Di-ammonium phosphate is between 5 to 55mM and d-ephedrine concentration is in range between 5 to 500mM.

In accordance to one more embodiment, the sonication frequency is between10 Hz to 100 Hz and specifically inoculum to substrate ratios (ISRs) used for reaction is between 0.1 to 3.

DETAILED DESCRIPTION OF THE INVENTION

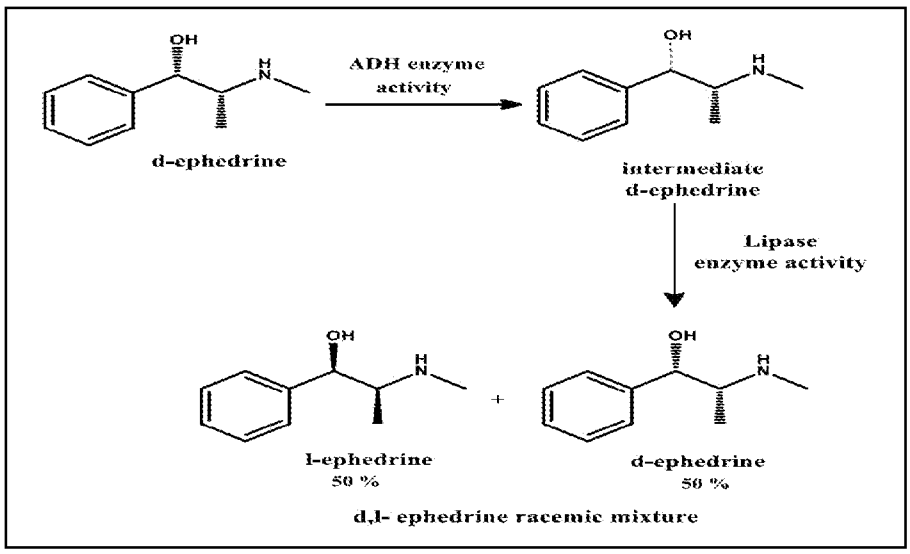
This invention relates to process for racemization of dextro-rotatory ephedrine via enzymatic kinetic conversion, and is particularly useful for conversion of dextro-rotatory ephedrine (d- ephedrine) to racemic mixture of dextro-rotatory ephedrine

(d-ephedrine) and levo-rotatory ephedrine (l-ephedrine) that exhibit potential bronchodilatory and anti-hypotensive activities.

The synthetic production of racemic ephedrine results into formation of detro-rotatory ephedrine (d-ephedrine) along with the levorotatory ephedrine (l-ephedrine), of which the latter one, l-form is comparatively a more valuable compound. The d-form cannot be utilized in pharmaceutical companies and thus remains as an undesirable compound. By racemizing the d-ephedrine and further subjecting it to any of the known technique for resolution of racemic mixture, it is possible to recover l-ephedrine again from it.

Hence, the present invention is a greener approach for utilizing the undesirable d-ephedrine produced during synthesis into a desirable l-ephedrine by developing a simple process using biocatalysis phenomena.

According to the present invention, the process is developed to convert d-ephedrine into racemic mixture of d- and l-ephedrine. This racemization required the inversion of functional groups present on the chiral carbon atom of d-ephedrine molecule. The overall reaction is described in scheme 2 as follows;



SCHEME 2

Accordingly, the suitable strain of fungi, *Rhizopus oryzae* selected for biocatalysis as the racemase source for conversion of d-ephedrine into d,l-ephedrine which was procured from National Collection for Industrially Important Microorganisms (NCIM), Pune.

In one of the embodiment, the treated whole cells of the organisms were incubated with the substrate for certain period of time which resulted into total racemization of d-ephedrine (mixture of 50% d- ephedrine and 50% l-ephedrine).

The said invention of biocatalytic process for racemization of d-ephedrine is very simple, environmental friendly and highly economic process which can be easily scaled up for industrial use.

The suitable culture media helps in expressing the particular enzymes in whole cells for racemization of d-ephedrine. Pure racemic d and l-ephedrine is obtained without formation of other undesired by-products like pseudo-ephedrine. The total amount of d-ephedrine introduced in bio-reactor is totally racemized into d, l-ephedrine respectively. Hence the problem of batch variation is not significant even though using the treated whole cell enzymes instead of specific extracted and purified enzymes. Thus, it can prove to be an easily scalable, cost-effective and a green process for racemization of d-ephedrine.

Preparation of Biocatalyst:

The fungal strain was subcultured, grown, and the enzymes that are responsible for racemization were expressed in suitable media which can be grouped into three parts: Part I: Potato Dextrose Agar (PDA) Media, potato dextrose 0.05 to 1 g, Agar 0.05 to 1 g and de-ionized water. Part II: Solid media: 0.4 to 10 % glucose or sucrose or galactose and other such nutrients can be used, 1 to 5 % yeast extract or malt extract, 0.05 to 1 % peptone or other substrates can be used, of which peptone gave good results and 1 to 10 % agar. Part III: Liquid media: 0.5 to 10 % glucose or galactose or starch or sucrose and other similar nutrient compounds can be used, 0.05 to 2 % yeast extract or beef extract or malt extract can be taken, 0.01 to 2 % sodium chloride or potassium chloride or similar salts can be used and about 0.01 to 5 % tryptone or casein peptone can be taken.

Strains of *R. Oryzae* were first subcultured and grown on PDA slant from 5 h up to 5 days at around 20 to 40 ºC on petri plate in an aseptic area, followed by preparation of solid media. The media components were autoclaved and poured into the petri plates. Then, it was streaked on the solid media and kept for incubation to produce spores from 5 h to 7 days at 20 to 40 ºC. Finally, these spores were inoculated into the liquid media (autoclaved) for the microbial growth, in a conical flask. This flask was then placed in a rotary shaker at around 25 to 60 ºC and from 0 to 200 rpm for about 6 h to 10 days. The whole cells of *R. Oryzae* were harvested after formation of spore cells from liquid media by centrifugation, washed with distilled water and stored at 4 ºC and all other parameters required for enzymes to remain inactive during storage period.

Procedure for the biocatalytic racemization of d-ephedrine: For the racemization, about 0.02 to 5 g of wet whole cells of *R. oryzae* showing lipase enzyme activity of approximately about 40 U/mg and Alcohol dehydrogenase enzyme of about 170 U/mg were introduced in a flask containing di-ammonium phosphate buffer (10 mM, pH= 6 to 8). The reaction can be carried out in other similar buffers or in the water as well in the pH range of 5 to 9. Pre-treatment of the whole cells of the fungal strain was essential for cell disruption to obtain the intracellular active enzymes. Use of surfactants (TritonX, Tween20, etc.), homogenization, osmotic shock, ultrasonication and various other well-known techniques are followed for the cell disruption. The fungal strain in our case has a thick cell wall made up of chitin and the homogenization or osmotic shock would not give the desired results. Also, though surfactants would produce good results, it had its own drawbacks as it has the tendency to mix up with the substrate in the solution and it may also interfere in the analysis of the reaction sample using HPLC. Thus, ultrasonication was found to be the reliable method of pre-treatment to have access to the active intracellular enzymes from the whole cells of the strain required for the reaction. Few of which can be used in present case wherein, ultrasonication of the whole cells produced good results. This was then divided into 3 parts as follows:

Part I: The cells were treated with ultrasonication and the broth was kept as a control for incubation;

Part II: The cells were untreated with ultrasonication, the substrate d-ephedrine was added and kept as another control for incubation;

Part III: The cells were treated with ultrasonication, the substrate d-ephedrine was added and kept as reaction mass for incubation;

All three sets were kept in rotating shaker at about 20 to 60 °C, 0 to 200 rpm and from 6 h to 7 days. The analysis was carried out using chiral HPLC column chromatography. Preparation of di-ammonium phosphate buffer was done by adding 1 mM of octanoic acid in 10 mM di-ammonium phosphate as dibasic buffer, pH-6.9 (o-phosphoric acid was used to vary the pH). Chiral column used for the method was Diacel chiralpak AGP (10 cm X 4.0 mm X 5 μm) at column temperature: 25 °C flow rate: 0.9 mL/min, absorbance wavelength: 215 nm. The standard peaks of d-ephedrine and d,l-ephedrine were obtained. The racemic d,l-ephedrine on the chiral column gave two different resolved peaks of d- and l-ephedrine by the given method of analysis. Then the reaction samples were analysed with the same developed method.

The reaction was carried out for different concentrations of substrate d- ephedrine and was studied for racemization. The total racemization of d- ephedrine was obtained which gave up to 50-50 ratio of d- ephedrine and l- ephedrine, respectively.

PREPARATION EXAMPLES:

EXAMPLES

Example 1: Racemization using Chemical catalysis- Racemization of d-ephedrine using modified heterogeneous nanocatalysts, external hydrogen pressure and different solvents:

Synthesis of Pd/AlO(OH) as racemizing catalyst was done to use the strategy of dynamic kinetic resolution studies of d-ephedrine. The reported method for synthesis of Pd/AlO(OH) was slightly modified using solvothermal treatment to obtain fine nanocatalyst powder.

$PdCl_2$ (200 mg, 2.3 mmol), pluronic P123 (4.0 g) and absolute ethanol (10 mL) were added together in a teflon coated bomb reactor. Al(O-sec.-Bu)3 (9.1 g, 37

mmol) was added carefully to the above mixture. The reactor was then tightly closed and placed in oven. It was subjected to thermal heating at 160 °C for about 3 h. It was then cooled at room temperature, decanted and the solid was then filtered and washed with acetone twice and dried at 120 °C for 2 h. ash coloured powder was obtained (3 g, 3.2 wt % of Pd).

Experiments : The catalyst 3.2 % Pd/AlO(OH) and reactant d-ephedrine were taken in solvent (upto 30 mL) and poured in autoclave reactor (100 mL capacity, Amar equipments). The autoclave was flushed 3 times and finally purged with H_2 pressure (about 20 bar) and maintained throughout the reaction. The temperature and agitation speed were also tuned according to the required experimental conditions.

Detection Method: The chiral HPLC method was developed for the separation of the d and l enantiomer from the racemic mixture of the dl-ephedrine as well as for the d-ephedrine standard in water.

Chiral column: Diacel chiralpak AGP (10 cm X 4.0 mm X 5 μm)

Column temperature: 25 °C

Flow rate: 0.9 mL/min

Mobile phase: 1 mM of octanoic acid in 10 mM di-ammonium phosphate as dibasic buffer, pH-6.9

Absorbance wavelength: 215 nm

An experiments were carried out in autoclave reactor (100 mL capacity, Amar equipments) for dynamic kinetic resolution studies:-

Table 1: Reactions carried out using 3.2% Pd/AlO(OH) catalyst

Sr No	Solvent	Catalyst modifiers	Experimental conditions			Result
			T (°C)	P (bar)	Rpm	
1.	1,4-dioxane		70	20	800	No product obtained
2.	water		70	20	800	d,l- ephedrine racemization obtained (4h) 80:20 ratio
3.	water		70	-	800	No product formed
4.	water		90	20	800	No product formed
5.	methanol		70	20	800	d,l- ephedrine racemization obtained (4h) 80:20 ratio
6.	water	l-tartaric acid	70	20	800	No significance observed
7.	IPA		70	-	500	No product formed
8.	IPA	laccase	70	-	500	No product formed
9.	IPA	laccase + l-TA	70	-	500	No product formed

Table 2: Reactions carried out using 0.5% Pt/alumina catalyst

5

Sr No	Solvent	Catalyst modifiers	Experimental conditions			Result
			T (°C)	P (bar)	Rpm	
1.	1,4-dioxane	cinchonidine	70	20	800	No racemization
2.	water	cinchonidine	70	20	800	No racemization
3.	methanol	cinchonidine	70	20	800	No racemization

Result: Racemization of d-ephedrine was observed to some extent (80% d-ephedrine and 20 % l-ephedrine)after 4 h in water as solvent, with 3.2 % Pd/AlO(OH) catalyst at temperature 70 °C, H_2 pressure 20 bar and agitation speed 800 rpm The reaction was further kept constant and samples were taken after 8 h, 12 h, 24 h which showed that there was no further change in concentration of l-ephedrine formed as that at 4 h.

On removal of external hydrogen pressure there was no racemization of d-ephedrine observed. Also, increasing temperature to 90 °C showed no product formation which may be due to its action on catalyst. Few repeated reactions were carried out which followed the same trend. Addition of l-tartaric acid showed no significance in the reaction. Solvent also played important role in this system.

Conclusion: The catalyst Pd/AlO(OH) shows conversion of d-ephedrine to d,l-ephedrine to some extent in water as solvent. No product was formed on increasing the temperature of the reaction at 4 h. Role of solvent in the system is also important.

Example 2: Enzyme catalysis- Whole cell biocatalytic racemization of d-ephedrine to l-ephedrine using Rhizopus Oryzae.

Step1: The cultures of Rhizopus species, Aspergillus species and Escherichia coli were selected, subcultured and stored in refrigerator at 4 °C which can be used as an enzymatic whole cell biocatalyst for bioconversion of racemic ephedrine. The fungal and bacterial cultures were maintained on suitable growth media.

Step2: Preparation of Whole cell Biocatalyst (Racemization catalyst)

i. Rhizopus Oryzae which can be used in enantioselective production were grown in solid medium containing 2% glucose, 2% yeast extract, 0.1% Peptone and 2% Agar

ii. The plates were incubated at 30ºC for 3-4 days for spore production.

iii. Spores were inoculated in liquid medium containing 2% glucose, 0.5% yeast extract, 0.5% sodium chloride, 1% tryptone and was grown in a rotary shaker at 30ºC for 2 days.

iv. After 2 days, the culture media were centrifuged and washed for 2-3 times with distilled water. The supernatant containing media was discarded and the pellet was suspended in 10mM Diammonium phosphate buffer, pH=6.9 (100 ml).

v. These whole cell suspension were ultrasonicated in 5 to 10 seconds interval from 30 seconds to 5 minutes which can be used as a whole cell enzyme. Also, 0.5% of Tween 80 was used as a whole cell disruption method.

Step3: Whole cell Biotransformation

i. 25mM of d-ephedrine were added to each ultrasonicated treated whole cell suspension

ii. The reaction was carried out on an orbital shaker at 30°C for 24 hrs.

iii. After the reaction was completed, the fungal biomass was centrifuged and then filtered which was monitored by HPLC.

Example 3: Weight variation of Substrates:

A. 10 mg d-ephedrine was added in 10 mL of buffer solution along with ultrasonicated whole cells and kept for incubation at 20- 60 °C, from 0 to 200 rpm for 6 h to 5 days. Samples were collected at every 6 h reaction time. It was observed that the reaction samples gave total racemic mixture of d- and l- ephedrine (50:50) at 36 h.

B. 16 mg d-ephedrine was added in 10 mL of buffer solution along with ultrasonicated whole cells and kept for incubation at 20- 60 °C, from 0 to 200 rpm for 6 h to 5 days. Samples were collected at every 6 h reaction time. It was observed that the reaction samples gave total racemic mixture of d- and l- ephedrine (50:50) at 24 h.

C. 20 mg d-ephedrine was added in 10 mL of buffer solution along with ultrasonicated whole cells and kept for incubation at 20- 60 °C, from 0 to 200 rpm for 6 h to 5 days. Samples were collected at every 6 h reaction time. It was observed

that the reaction samples gave total racemic mixture of d- and l- ephedrine (50:50) at 18 h.

D. 25 mg d-ephedrine was added in 10 mL of buffer solution along with ultrasonicated whole cells and kept for incubation at 20- 60 ºC, from 0 to 200 rpm for 6 h to 5 days. Samples were collected at every 6 h reaction time. It was observed that the reaction samples gave total racemic mixture of d- and l- ephedrine (50:50) at 12 h.

E. 50 mg d-ephedrine was added in 10 mL of buffer solution along with ultrasonicated whole cells and kept for incubation at 20- 60 ºC, from 0 to 200 rpm for 6 h to 5 days. Samples were collected at every 6 h reaction time. It was observed that the reaction samples gave total racemic mixture of d- and l- ephedrine (50:50) at 12 h.

F. 100 mg d-ephedrine was added in 10 mL of buffer solution along with ultrasonicated whole cells and kept for incubation at 20- 60 ºC, from 0 to 200 rpm for 6 h to 5 days. Samples were collected at every 6 h reaction time. It was observed that the reaction samples gave total racemic mixture of d- and l- ephedrine (50:50) at 12 h.

G. 1000 mg d-ephedrine was added in 100 mL of buffer solution along with ultrasonicated whole cells and kept for incubation at 20- 60 ºC, from 0 to 200 rpm for 6 h to 5 days. Samples were collected at every 6 h reaction time. It was observed that the reaction samples gave total racemic mixture of d- and l- ephedrine (50:50) at 12 h.

In example 3 (A) to (G) the concentration of the substrate is varied while keeping the buffer volume same. In example 3(G) we tried to increase the volume of buffer along with the substrate concentration. The dextro- rotatory ephedrine gets racemized into detro- and levo- rotatory ephedrine in 12 h.

Example 4: Effect of sonication and Surfactant: A control experiment was performed using whole cell microorganisms with the pre-treatment.

Ultrasonicated samples :

- Whole cell control showed no changes on ephedrine

- 3 min ultrasonicated sample showed racemization of d-ephedrine
- 5 min ultrasonicated sample showed formation of some undesirable unknown product.
- 10 min ultrasonication might have produced enzyme denaturation to give undesired results.

Tween 80 treated samples :

 0.5 % Tween 80 treated samples showed racemization of d-ephedrine

But it also possess the tendency to mix up with the substrate, thus creating problems in separation and purification of the product.

Conclusion: Ultrasonic action in a whole cell suspension causes cell disruption and conformational changes in protein structure of enzymes. These conformational structure may cause increase in enantioselectivity of ephedrine. Tween 80 is also used for disruption and dispersion of fungal cultures for release of enzymes but it also mix up with the substrate and creates problem in separation and purification of desired compound.

Example 5: Effect of different strains on reaction yield:

Different fungal strains were used for racemization.

The comparison study of different species of fungal strains for racimization of d-ephedrine (10mg/10 mL) was carried out for 24 h reaction time.

About 1 g of wet whole cells of the each strain were transferred to Diammonium phosphate (DAP) buffer. The following sets of reactions were performed on d-ephedrine.

Pretreatment of whole cell: The whole cells of the strain were disrupted using ultrasonication in the buffer for the release of intracellular enzymes. This pretreated whole cells were further subjected for reactions.

1) Broth Control (3 min pretreated whole cell + 10mM DAP Buffer) 2) Ephedrine Control (d-ephedrine + 10 mM DAP Buffer) 3) Reaction time: 24 h (3 min pretreated whole cell + 10mM DAP Buffer + d-ephedrine)

The above reactions were kept in a rotating shaker at 30^{0}C.

Table 3: Interpretation of HPLC % area conversion data for d-ephedrine racemization where, '+': d-ephedrine, '-': l-ephedrine.

Strains	% area at 0 h	% area at 24 h
R. oryzae	+100	+50, -50
A.oryzae	+100	+100
R. oligosporous	+100	+100
R. stolonifer	+100	+100

Observations : Racemization of d-ephedrine was achieved successfully in buffer using pretreated whole cells of *R. oryzae.* While *Aspergillus oryzae, R. oligosporous, R. stolonifer* had no effect on d-ephedrine even after 24 h of reaction time.

Conclusion : The specific species of *Rhizopus* species Strains give racemization of d-ephedrine.

Example 6: Effect of Enzyme Activity in reaction mass: The fungal strain consists of a large number of enzymes of which only few enzymes are active and take part in the reaction. Lipase enzyme and Alcohol Dehydrogenase (ADH) generally playing role in racemization of d-ephedrine. This study was carried to identify the concentration of enzymes required particularly for racemization of d-ephedrine.

A. Lipase Activity

- The lipase activity of both the homogenized and unhomogenised culture was determined by the pH stat method.
- 50 mL of homogenized culture was added to 10 mL of Tris-HCl buffer (1mM, pH =7.2) and then 200 µL of tributrin was added as a substrate.
- The reaction was performed at 40 ºC, pH 7 and 200 rpm for 10 min.
- Then, a mixture (10 mL) of acetone–ethanol (1:1, v/v) was added to the reaction mixture to denature the enzyme.
- Phenolphthalein indicator was added and the mixture was titrated against the standard 0.1 N NaOH solution.
- The titration of cell suspension was carried out at specific interval of time for about 12 h reaction.

Table 4: Lipase activity (U/mg) obtained for particular time (h)

incubation time (h)	lipase activity (U/mg)
1	15
2	29.1
3	33.3
4	34.5
5	34.6
6	34.7
7	35
8	35
9	35.1
10	37.6
11	39.4
12	39.5

B. Alcohol dehydrogenase (ADH) activity:

- ADH activity was analyzed spectroscopically by measuring the increase in absorbance at 340 nm promoted by formation of NADH during oxidation of ethanol.

- Only liquid part of homogenized cell suspension was used in ADH activity. These cell suspensions were filtered with appropriate filter.
- ADH activity was measured in 3 mL cuvette containing 50 µL of 20 mM ethanol, 1 mL of distilled water and 1.25 mL of 13 mM β-NAD.
- Reaction starts by the addition of 200 mL of cell suspension.
  The spectrophotometric absorbance of cell suspension was carried out at specific interval time for about 12 h reaction.

Table 5: ADH activity (U/mg) obtained for particular time (h)

Incubation time (h)	ADH activity (U/mg)
1	33
2	45
3	59
4	61
5	67
6	72
7	79
8	82
9	84
10	90
11	120
12	170

Example 7: Detection of undesired by-product like pseudo-ephedrine in product

To confirm for any possibility of pseudoephedrine formation in the reaction mass, studies were carried out as follows,

Samples to be analyzed,

i. d,l- ephedrine standard (1000 ppm)
ii. pseudo-ephedrine standard (1000 ppm)
iii. Reaction sample (10,000 ppm)
iv. Reaction sample + d,l- ephedrine standard (50:50)

v. Reaction sample + pseudo-ephedrine standard (50:50)

Method of analysis was HPLC detection technique:

Mobile phase: 940 ml of buffer and 60 ml of methanol, filter through 0.45µ filter and degas.

Buffer: Weigh accurately 11.6 g of ammonium acetate and dissolve and dilute to 1000 ml with water. Adjust to pH 4.0 ± 0.01 with glacial acetic acid.

Chromatography Conditions:

- Instrument : Agilent 1100
- Column : **4.6 mm X 250 mm, ZORBAX Eclipse XBD-Phenyl**
- Packing : **5 µm** particle size, Spherical
- Flow rate : 1.0 ml per minute
- Wavelength : 257 nm
- Injection Volume : 20 µl

Observation: There was no detection of pseudo-ephedrine peak in Reaction sample when compared with chromatograph and retention of pseudo-ephedrine standard.

CLAIMS

I Claim,

1. A process for Racemization of dextro- rotatory ephedrine, the process comprising of:

Step 1: Preparation of suspension of *Rhizopus Oryzae* fungi pellets in diammonium phosphate buffer having pH in the range of pH 5 to 9,

Step 2: Ultrasonicating the step 1 suspension for time between 3 to 10 min,

Step 3: Addition of d-ephedrine in ultrasonicated suspension of step 2) and carrying out reaction at temperature between 25 to 35 °C for 10 to 36 h under constant stirring,

Step 4: Separation of liquid from solid mass by centrifugation at 300 to 10000 rpm.

2. The process for racemization of dextro- rotatory ephedrine as claimed in claim 1, wherein concentration of Di-ammonium phosphate is between 5 to 55mM.

3. The process for racemization of dextro- rotatory ephedrine as claimed in claim 1, wherein substrate d-ephedrine concentration is in range between 5 to 500mM.

4. The process for racemization of dextro- rotatory ephedrine as claimed in claim 1, wherein sonication frequency is between 10 Hz to 100 Hz.

5. The process as claimed in claim 1, wherein inoculum to substrate ratios (ISRs) used for reaction is between 0.1 to 3.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN2019/050797

A. CLASSIFICATION OF SUBJECT MATTER
C07C215/08,C07C209/68,C12P7/02,C12P41/00 Version=2020.01

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)

C07C, 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)

TotalPatent One, IPO Internal Database

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2007090767 A1 (BASF AKTIENGESELLSCHAFT) 16 August 2007 (16-08-2007) Claims 1-9; Abstract	1-5
A	US 2214034 A (LUNAMED AG) 10 September 1940 (10-09-1940) Claims 1-5; Examples	1-5
A	WO 8909765 A1 (SEPRACOR, INC.) 19 October 1989 (19-10-1989) Abstract, Claims 1-10, 32; Examples	1-5

☐ Further documents are listed in the continuation of Box C. ☒ 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
"D" document cited by the applicant in the international application
"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
07-02-2020	07-02-2020
Name and mailing address of the ISA/ Indian Patent Office Plot No.32, Sector 14,Dwarka,New Delhi-110075 Facsimile No.	Authorized officer Koushik Pal Telephone No. +91-1125300200

Form PCT/ISA/210 (second sheet) (July 2019)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/IN2019/050797

Citation	Pub.Date	Family	Pub.Date
WO 2007090767 A1	16-08-2007	CN 101379193 B	26-12-2012
		US 8153408 B2	10-04-2012
		EP 1984515 B1	01-09-2010
		JP 5209501 B2	12-06-2013
		DE 602007008840 D1	14-10-2010
WO 8909765 A1	19-10-1989	JP 03504599 A	09-10-1991
		EP 423133 B1	20-09-1995
		US 5057427 A	15-10-1991
		KR 19970005052 B1	11-04-1997
		CA 1266248 A1	27-02-1990