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DEVELOPMENT OF AN IMPROVED SELECTIVE ENZYMATIC PROCESS FOR DARUNAVIR INTERMEDIATE

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ABSTRACT

The manuscript describes the concise, efficient and cost effective route of synthesis of (3R, 3aS, and 6aR)-hexahydrofuro [2, 3-b] furan-3-yl acetate, a key unit for an antiretroviral medication, Darunavir (DRV), used for the treatment and prevention of HIV/AIDS. The process involves the use of an immobilized enzyme to circumvent tedious work up problems like celitefiltration, giving away the final product with higher yield and purity. The investigational studies done in this article will surely aid in improving the existing processes in many aspects as discussed above.

KEYWORDS

Enzymatic process, Concise and Darunavir.

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INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) produced by Human immunodeficiency virus (HIV) is a deteriorating ailment of the immune system and has become an irresistible medical distress worldwide. Darunavir, sold under the brand name *Prezista*, enumerated as WHO's vital medicine, is FDA approved (2008) and is the most effective and safe medicine for the treatment in HIV AIDS. It was approved for therapeutic use in the United States in 2006[1]. It is an OARAC (The Office of AIDS Research Advisory Council) (DHHS) acclaimed treatment option for adults and

adolescents, irrespective of prior HIV treatment in the past[2].

Darunavir, a nonpeptidic inhibitor of protease (PR), was developed in a way to escalate its interactions with HIV-1 protease, through a strong interaction between Darunavir and active site of PR through a number of hydrogen bonds[3]. Thus, more resistant against HIV-1 protease mutations. Additionally, the mainstay of HIV-1 protease upholds its 3-D conformation in the presence of mutations. Since Darunavir networks with this steady portion of the protease, there is lesser chance of the disruption of this PR-drug interaction by a mutation[4].

(3R, 3aS, and 6aR)-hexahydrofuro [2, 3-b] furan-3-yl acetate is a common intermediate to chiral Darunavir and thus is of particular importance (Figure No.1)[5]. The current procedure of its preparation involves the use of liquid CAL-B enzyme which makes the workup procedure problematic as it implicates emulsion formation and difficult layer separation leading to necessitate celite filtration. Seeing the importance of Darunavir, it was felt worthwhile to further examine the means to prepare this compound.

MATERIAL AND METHODS

Enzymes

CAL-$B_{ex\ 10000}$ immobilized enzyme was purchased from Fermenta Biotech.

Chemical Reagents

Sodium di hydrogen phosphate mono hydrate was purchased from Avra, Sodium bicarbonate and sodium chloride were purchased from Merck. Dichloromethane was purchased from Spectrochem Pvt. Ltd.

Analytical Methods

Chiral Gas chromatography

Chiral gas chromatographic analysis of (3R, 3aS, and 6aR)-hexahydrofuro [2, 3-b] furan-3-yl acetate was performed on Agilent Technologies Gas chromatography instrument using ASTEC CHIRALDEX G-PN (30 m* 0.25mm* 0.12µm) column. The elution was carried out with nitrogen as carrier gas and the eluents were detected by Flame ionization detector. The retention time was 12.15 min for (3R, 3aS, and 6aR)-hexahydrofuro [2, 3-b] furan-3-yl acetate. The two diastereomers I and II were observed at retention times 11.7min and 11.9min respectively.

RESULTS AND DISCUSSION

In the reported process of synthesis of Darunavir, optically active (3R, 3aS, and 6aR)-hexahydrofuro [2, 3-b] furan-3-yl acetate2, is one of the key intermediates involving its reaction with Hexahydrofuro [2, 3-b] furan-3-yl acetate1 using liquid CAL-B enzyme leading to tiresome workup glitches (Scheme I). Liquid enzymes always pose a threat of serious emulsion formation with the reaction mixture, which may also result in final yield loss.

On the other hand, the same enzyme when used in an immobilized form not only solves the above mentioned road blocks but also can be recycled multiple times to decrease cost. Furthermore, immobilized enzymes are more active when compared to their liquid counterparts, and thus lesser quantity used also facilitates ease of handling in scale-up processes.

The same immobilized enzyme (CAL-B_{ex} 10000) was successfully and practically recycled for 11 continuous cycles without any loss of activity in the enzyme. The yield and purity obtained were consistent with each passing cycle. The reaction maintenance time also did not get affected during the subsequent recycles and most importantly, the reactions complied with the specifications till the very end of the 11^{th} cycle. The immobilized enzyme was carefully stored in 2-8ºC when and if required.

To preserve the integrity of immobilized enzymes so that it survives multiple cycles of re-use, the following points are to be implemented:

Slow stirrer speed

This prevents the enzyme beads from breaking and creating enzyme debris that might be difficult to separate during reaction work up.

Proper enzyme storage conditions are to be followed

The used enzyme is to be stored in 2-8ºC in a buffer solution of pH 7-7.5. This way enzyme activity is preserved for longer periods of time.

A buffer solution should be incorporated in the reaction mixture

A buffered reaction system has a lesser pH gradient. This way the enzyme activity is retained in the reaction mixture.

The reactions were performed in regular round bottom flasks with blades as well as Rotating Bed Reactors. The Rotating Bed Reactors have an advantage over RBFs. The immobilized enzyme beads in them are well protected in a porous mesh as opposed to the threat of being broken down by the paddles of the regular stirrers.

Hence the immobilized beads are resistant to breakage even after multiple re-cycles in the former reactors. The rotating bed reactors involve rapid aspiration of the reaction solution from the vessel and percolates it in and out of the immobilized enzyme bed. Even though the reaction maintenance time was seen to increase in the Spinchem reactor reactions, the integrity of the enzyme beads was preserved.

However, it was observed that in RBF recycle reactions, even though the enzyme beads were broken down to powder like appearance, it did not have any significant effect on its activity and the overall reaction maintenance time.

Thus, in an effort to develop an improved, abridged, cost effective and convenient selective route for the synthesis of (3R, 3aS, and 6aR)-hexahydrofuro [2, 3-b] furan-3-yl acetate, immobilized enzyme was used that resulted in lowered enzyme input quantity, evadedcelite filtration and resulted in no or minimal workup snags and saved overall workup time. It also helped in yielding higher yields and chiral purity as high as 100%. The said process for the same from our lab will be discussed in detail in this manuscript (Table No.2).

Hexahydrofuro [2, 3-b] furan-3-yl acetate1 was taken in sodium dihydrogen phosphate buffer solution (pH-6.0-6.5), which was loaded with enzyme (CAL-B), stirred at 37-43°C and maintained at pH-6.0-6.5. After completion of the reaction, product was extracted with dichloromethane, followed by work up of the reaction mass and distillation of the organic layer to get darunavir intermediate as the final product 2.

It was seen that when commercial starting material of around 89% purity was used, the product purity obtained was 88% as against the purified starting material of 98% that yielded a 99% pure product. The following by-products that were present in the commercial starting material, did not affect the chiral purity of the final product and had negligible effect on the yield and enzyme activity as well.

Critical process parameters and their control

Variation in Enzyme (type and quantity) - Optimization and feasibility

The investigational studies involved the use of enzymes like *Novozym 435*, *Novozym 51032* and *CAL-B Immobilized* (5% and 10%). For synthesis of Darunavir intermediate, it was found that *CAL-B (Immobilized)* 10% proved to be the best with respect to the yield obtained (0.33 w/w) and overall reaction maintenance time (27h).

Variation in pH and temperature

1 was taken in two different sodium dihydrogen phosphate mono hydrate buffer solutions i.e. one having pH-5.0-5.5 and another with pH 6.5-7.0, which was loaded with enzyme (CAL-B), stirred at 37-43°C, maintaining the pH-5.0-5.5 and 6.5-7.0 respectively. All the other reaction parameters remained same during the experiment. It was observed that when the experiment was performed at pH-5.0-5.5 instead of 6.0-6.5 (0.35 w/w), reaction compliance time increased from 24h to 30hand in 24h when pH was pH-6.5-7.0 instead of 6.0-6.5 reaction complies with in 24h.No risk was involved when the reaction was performed by maintaining the pH-5.0-5.5 (0.27 w/w) and pH-6.5-7.0 but yield obtained was on the lower side (0.25 w/w).

In another experiment, the reaction temperature was varied i.e. at 34-36°C and at 43-45°C instead of 37-43°C keeping all the other parameters same. It was observed that when experiment was performed at 34-36°C instead of 37-43°C reaction compliance time increased from 24h to 28h. However, when experiment was performed at 43-45°C instead of 37-43°C, there was no change in the Yield and/or purity.

Variation in Reaction time

Our optimization studies of the reaction conditions contain the variation in the reaction time up to 120h

instead of 24h. This study revealed that there was no change in purity (99.87%) but resultant yield was lower (0.26 w/w).

Variations in Workup process

Another potential means to avoid the lengthy workup procedure is to by**p**ass vacuum distillation. It was observed that there was no change in yield and purity when the experiment was performed without vacuum distillation. In an attempt to reduce the volume of MDC (Dichloromethane) used in the workup process, 15 volumes was reduced to 10 volumes. This showed no significant change was seen in purity but the yield obtained was on the lower side (0.28 w/w).

EXPERIMENTAL

Procedure for the selective preparation of (3R, 3aS, and 6aR)-hexahydrofuro [2, 3-b] furan-3-yl acetate 2

In a flask, charged water (50 volumes) and sodium dihydrogen ortho phosphate mono hydrate (1.13 mole eq.). Stir and adjust the pH of the buffer solution to 6.0-6.5 by using 7% sodium bi carbonate solution (Prepare using 70.0 g Sodium bicarbonate dissolved in 1000 ml of water).

It is noted that during pH adjustment, foaming was observed. Stir and charge starting material 1 (1.0 mole eq.) followed by 10% CAL-B (Immobilized). Then the temperature of the reaction was raised to 40ºC, stirred and maintained the pH 6.0 to 6.5 by using 7% sodium bicarbonate solution. (Meanwhile monitor the reaction by GC to check enantiomer and diastereomers limits: Enantiomer-NMT 0.05% and Total diastereomers is NMT 0.50%). Cooled the reaction mass and filtered the enzyme, washed the enzyme with water. Dried under vacuum in nitrogen atmosphere, stored that enzyme in water and used the same enzyme for next reaction up to four recycles. Then, charge dichloromethane (5 times) to the reaction mass. Stir, settle and separate the layers afterwards. Organic layer and aqueous layer collected in fresh containers. To the aqueous layer, added dichloromethane, stirred, settled and separated the layers. Combined organic layers was washed with 10% sodium chloride solution (prepare by using 50 g sodium chloride dissolved in 500 ml of DM water). The organic layer was then concentrated under vacuum till almost no solvents distills to obtain 2. Yield: 0.4 w/w, Mass (m+1): 172, Chiral Purity: 99.87%.

Table No.1: Recycle studies

S.No	Reactor used	Enzyme used	Chiral Purity (%)	Time (Hrs)	Yield(w/w)
1	Round bottom flask	Fresh enzyme	99.65	28hrs	0.33
2	Rotating Bed Reactor	Fresh enzyme	99.77	27hrs	0.32
3	Round bottom flask	Recycle enzyme	99.66	26hrs	0.34
4	Rotating Bed Reactor	Recycle enzyme	99.86	35hrs	0.30

Table No.2: Comparison of Critical Process Parameters for Liquid and Immobilized enzyme

S.No	Parameter	Liquid vs. Immobilized	
1	Enzyme Type used	Liquid	Immobilized
2	Quantity of enzyme used	33-40g (/100g)	10-20g (/100g)
3	Celite filtration	Required	Avoided
4	Work-up emulsion formation	Emulsion formed	No emulsion formed
5	Reaction time	80hrs	30hrs
6	Enzyme addition	In 5 intervals	All at once
7	Yield	0.28w/w	0.35w/w

Table No.3: Product purity comparisons with purified and crude starting material'

S.No	Starting material purity	Product Purity	Product Chiral purity
1	89%	88%	99.65%
2	98%	99%	99.99%

Table No.4: Different Enzymes used in the project

S.No	Enzyme used	Yield	Chiral Purity	Reaction time
1	Novozym 51032	0.21w/w	99.9%	24hrs
2	Novozym 435	0.3w/w	99.8%	57hrs

Table No.5: Optimization of pH and Temperature

S.No	Parameters	pH			Temp.		
1	-	Optimized pH (6-6.5)	5.0-5.5	6.5-7.0	Optimized temp (37-43)	34-36	43-45
2	Chiral Purity	99.84%	99.49%	99.72%	99.84%	99.81%	99.88%
3	Yield	0.35w/w	0.27w/w	0.25w/w	0.35w/w	0.31w/w	0.3w/w

Table No.6: Optimization of reaction maintenance time

S.No	Parameters	Reaction time	
1	-	Optimized time (24-30hrs)	120hrs
2	Chiral Purity	99.84%	99.87%
3	Yield	0.35w/w	0.26w/w

Table No.7: Optimization of the volume of solvent

S.No	Parameters	Solvent volume	
1	-	Optimized volume (15V)	10V
2	Chiral Purity	99.84%	99.76%
3	Yield	0.35w/w	0.28w/w

Table No.8: Variations in the Distillation process

S.No	Parameters	Distillation process	
1	-	With vacuum	Without vacuum
2	Chiral Purity	99.77%	99.84%
3	Yield	0.33w/w	0.35w/w

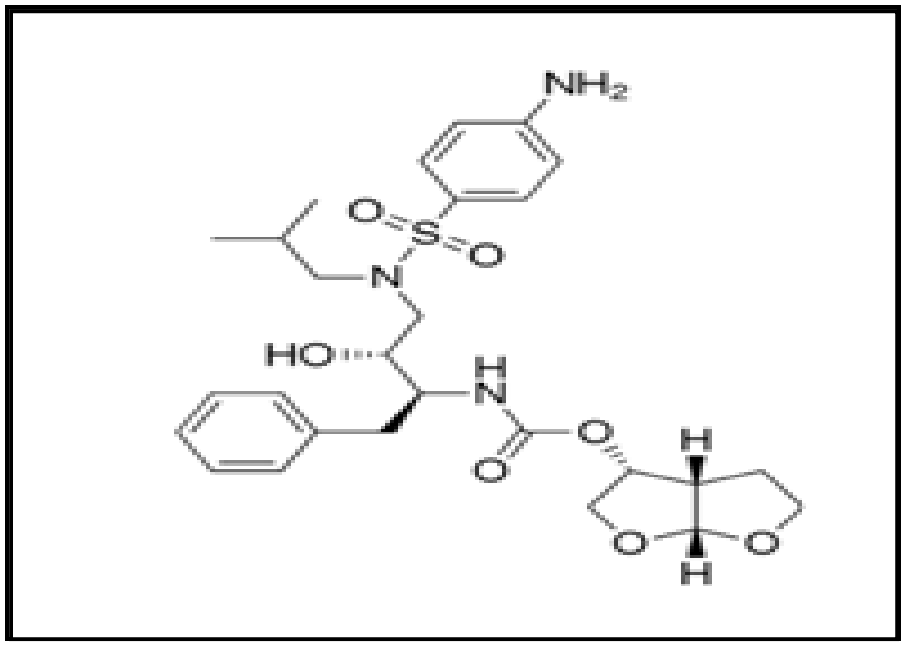
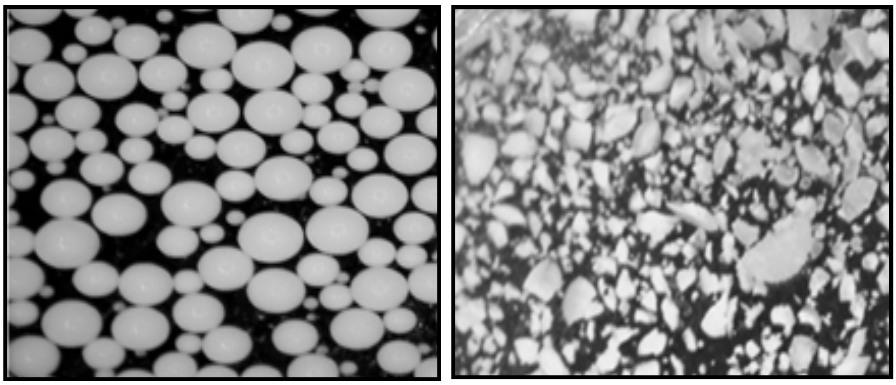


Figure No.1: Structure of Darunavir



(i) (ii)

Figure No.2: Enzyme microscopic images for conventional Stirrer (i) Before Reaction (ii) After Reaction

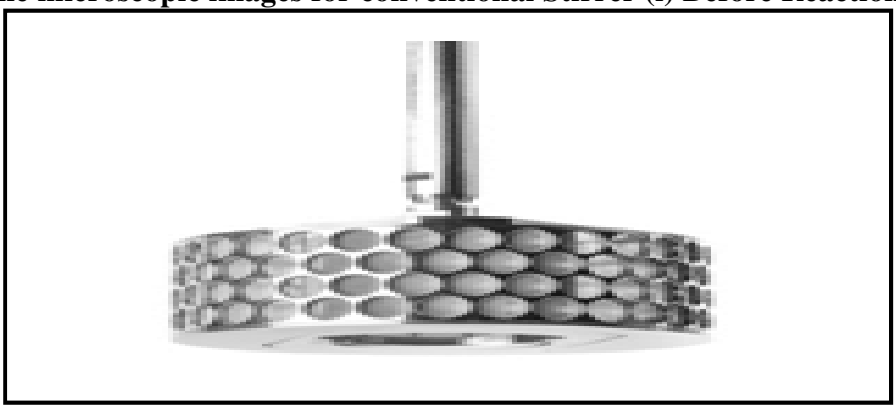
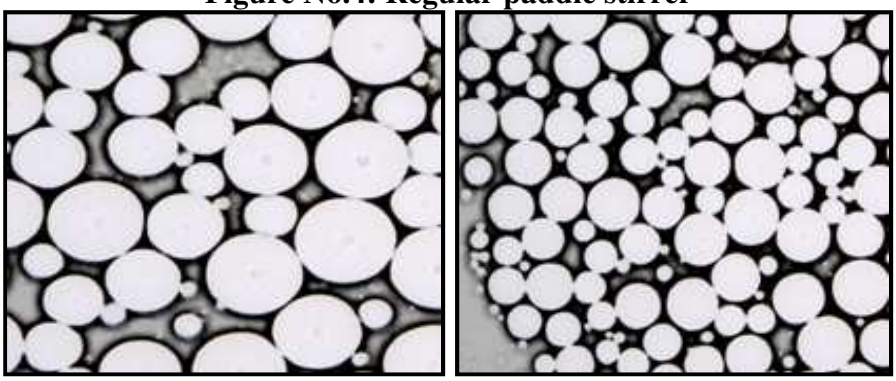


Figure No.3: Rotating bed reactor

Figure No.4: Regular paddle stirrer



(i) (ii)

Figure No.5: Enzyme microscopic images (i) Before Reaction (ii) After reaction

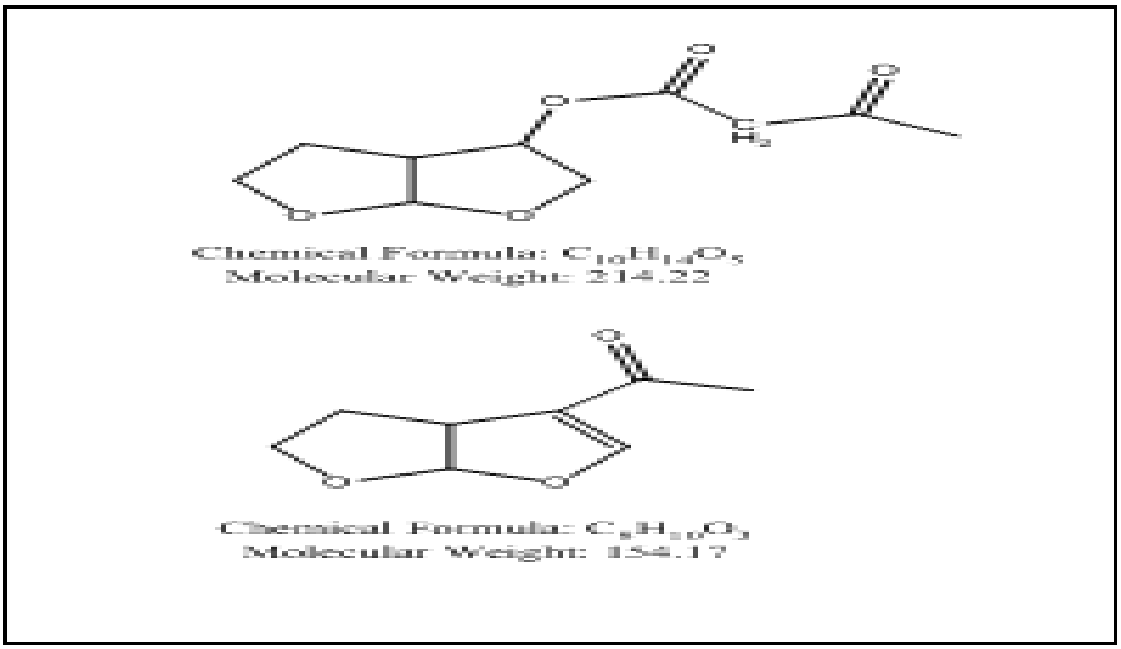
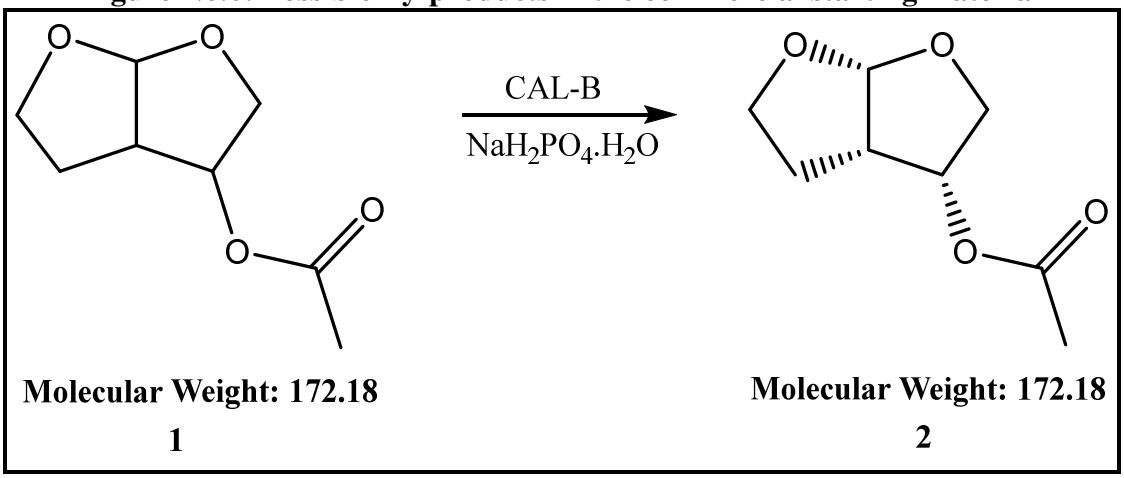
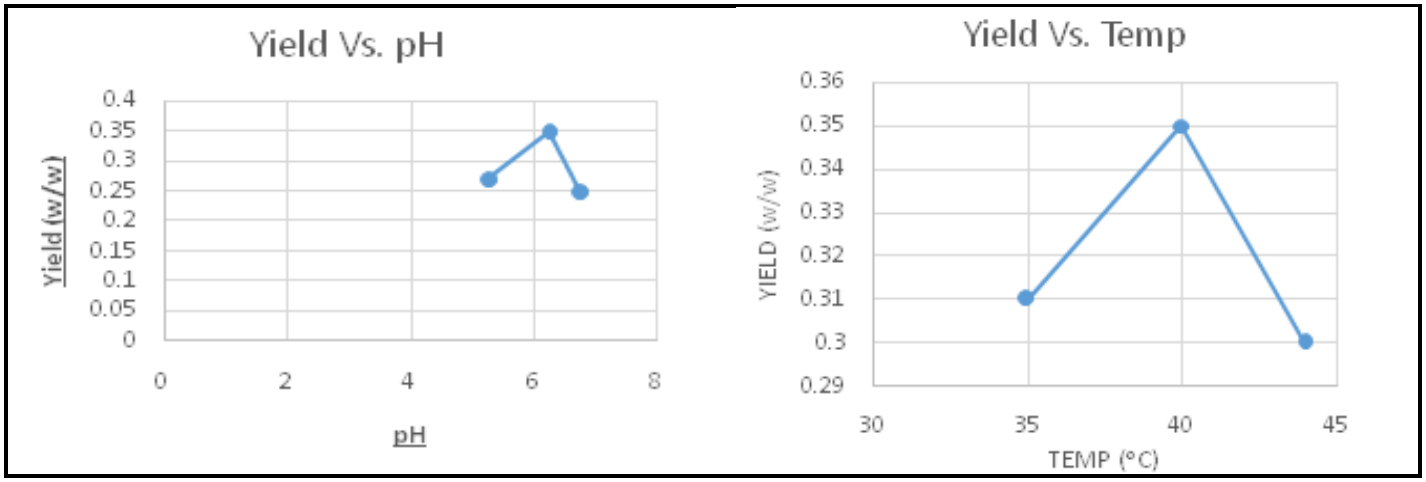


Figure No.6: Possible By-products in the commercial starting material



Scheme No.1



Graph No.1: Yield vs. pH **Graph No.2: Yield vs. Temperature**

CONCLUSION

Our investigation demonstrates a simple, efficient yet eco-friendly synthetic route involving a biodegradable enzymatic step for the synthesis of (3R, 3aS, and 6aR)-hexahydrofuro [2, 3-b] furan-3-yl acetate. This route of synthesis involves highly convenient chemical steps and is also a cost effective procedure. This process offers a new strategy and is best suitable for the preparation of (3R, 3aS, and 6aR)-hexahydrofuro [2, 3-b] furan-3-yl acetate and is also scalable in plant-level. Also, various practical insights are incorporated in this manuscript regarding handling and storage of immobilized enzymes to preserve enzyme activity over longer periods of time.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

BIBLIOGRAPHY

1. Sax P E. "FDA approval: Darunavir", *AIDS Clin Care,* 18(8), 2006, 71.
2. Guidelines for the Use of Antiretroviral Agents in HIV-1-Infected Adults and Adolescents, Developed by the DHHS Panel on Antiretroviral Guidelines for Adults and Adolescents - A Working Group of the Office of AIDS Research Advisory Council (OARAC), Full guidelines, November 3, 2008.
3. Nancy M. King, Moses Prabu-Jeyabalan, Ellen A. Nalivaika, Piet Wigerinck, Marie-Pierre de Bethune and Celia A. Schiffer. "Structural and Thermodynamic Basis for the Binding of TMC114, a Next-Generation Human Immunodeficiency Virus Type 1 Protease Inhibitor", *Journal of Virology,* 78(21), 2004, 12012-12021.
4. Ying Zhang, Yu-Chung E. Chang, John M. Louis, Yuan-Fang Wang, Robert W. Harrison and Irene T. Weber. "Structures of darunavir-resistant HIV-1 protease mutant reveal atypical binding of darunavir to wide open flaps", *ACS Chem Biol,* 9(6), 2014, 1351-1358.
5. "Process for the Preparation of Darunavir and Darunavir Intermediates", *Inventors: Michael Mizhiritskii, Rehovot (IL); Ehud Marom, Kfar Saba (IL), US 8,829,208B2,* 2014.

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