Bakers' Yeast Mediated Stereo Selective Reduction of Cis (+) Diketolactam to Cis (+) Hydroxylactam, a Key Intermediate for Diltiazem Synthesis in a Laboratory Scale Bioreactor

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***Abstract*—The reduction of (RS)-2-(4-methoxyphenyl)-l, 5-benzothiazepin-3,4(2H,5H)-dione[(RS)-1] to (2S,3S)-2,3-dihydro-3-hydroxy-2-(4-methoxyphenyl)-l,5-benzo-thiaepin-4(5H)-one using bakers' yeast was carried out in the laboratory scale 10L fermenter. Under optimum reaction conditions, 200g of substrate was reduced to desired chiral intermediate in 4L reaction volume with 79.33% of conversion within 89h. The optimum conditions for the effective reduction were at pH 6.5 and at 30 ℃ temperature. The crystals of desired isomer were isolated from the reaction mixture with the total yield of 42%, having 95.42% of assay purity with 99.80% of enantiomeric excess.**

***Index Terms*— Asymmetric reduction, bakers' yeast, bioconversion, diltizem hydrochloride.**

I. Introduction

Diltiazem hydrochloride is a representative calcium channel blocker that is used clinically as an effective antianginal and antihypertensive agent throughout the world. It is 1,5-benzothiazepine derivative with two asymmetric carbon atoms at C-2 and C-3 position. Among the possible diastereomers, the (2S,3S)-isomer exhibits strong coronary vasodialating activity. Therefore, stereoselective synthesis of the (2S,3S)-isomer has been attracting great attention. Commercially, diltiazam has been produced by a classical resolution process [1]. A number of studies have been reported on the stereoselective synthesis of diltiazem hydrochloride: chemical optical resolution [2]-[3], asymmetric dihydroxylation [4], asymmetric hydrocyanaton [5], Darzens reaction [6], Michael addition [7], asymmetric reduction [8] and enzymatic asymmetric hydrolysis [9] - [10]. In this process the yield of desired isomer does not exceed 50%, thereby generating enormous waste containing (2RS,3RS)-2-hydroxy-3-(4-methoxyphenyl)-3-(2-aminophenylthio) propionic acid. So, for favorable process economics, recycling of the waste enantiomer is essential. This was achieved by oxidizing the alcohol moiety (chiral centre) in the waste (mother liquor after resolution) to ketone, (*RS*)-2-(4-methoxyphenyl)-l,5-benzothiazepin-3,4(*2H,5H*)-dione and stereo specific reduction of ketone to form the desired(*2S,3S*)-2,3-dihydro-3-hydroxy-2-(4-methoxyphenyl) -l,5- benzo- thiaepin-4(*5H*)-one.

The chemo enzymatic synthesis of one of the diltizem chiral intermediate [(-)- MPGM] has been achieved by lipase mediated biocatalytic resolution of racemic *trans*-3-(4'-methoxyphenyl) glycidic acid methyl ester [(±)-MPGM] using immobilized enzyme system [11]. Stereo selective reduction of ketone to the desired isomer has been extensively studied from the viewpoint of their high efficiency. The chemical reduction of ketone using chiral reducing agent $NaBH_4$-(S)-(tert)-leucine has been reported to give only 86% yield. The biochemical asymmetric reduction of the prochiral ketone was reported [12] with a conversion yield of 92-94% using bakers' yeast in a small reaction volume of 3ml during a screening experiment. One-paper reports about the bakers' yeast mediated bioreduction of prochiral ketones using ethanol as an energy source [13]. It was further investigated for industrial application at shake flask level by which 100g of substrate was reduced in 1L reaction volume to produce desired intermediate in 80% yield [14]. Nevertheless there is no report on the bioreduction using a waste recycling process at flask or fermenter level for the production of Diltizem [15].

The present study deals with the optimization of bakers' yeast mediated stereo-selective reduction of acid waste to desired isomer in a laboratory scale fermenter. We have made an attempt to reduce 200g of substrate in 4L reaction volume using Meteoric- instant dry yeast as a source of bakers' yeast. The process conditions for the optimal bioconversion have been described and the desired isomer was isolated in pure form from the reaction mixture.

II. Materials & Methods

A. Strain

Bakers' instant dry yeast from Meteoric, Mumbai was

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procured and used for the study. We have screened different yeast strain for this biotransformation in the flask and finally selected Meteoric- instant dry yeast which showed better conversion, for the optimization study in fermenter.

B. Bioconversion Study in Shake Flask:

Initially the bioconversion was standardized in the shake flask with respect to the concentration of substrate, buffer pH and amount of yeast inoculum. The reaction mixture consists of 34ml of phosphate buffer and yeast inoculum, which was added to 1L Erlenmeyer flask and incubated on rotary shaker at 150rpm at 30 ℃. A 3.84ml of 50% dextrose monohydrate solution was fed to the flask after every 24h starting from zero hour. A substrate dissolved in 6ml of dimethyl formamide (DMF) was added to the reaction mixture with the intermittent addition of 500 µl at a time within 36h. After 120h, the reaction was terminated and the substrate and product was extracted as mentioned C.

C. Estimation of Product from Reaction Mixture:

10ml of reaction mixture was centrifuged at 5000rpm for 30min to separate biomass-containing product and supernatant was discarded. The sediment was extracted with 10ml of dichloromethane (CH_2Cl_2) and then centrifuged at 5000rpm for 30min to get the organic layer. The sediment was further extracted with same volume of CH_2Cl_2 and centrifuged. The organic layer was pooled together and concentrated to dryness using rotary evaporator at 45 ℃ and analyzed by HPLC as mentioned in D.

D. Analytical Methods:

HPLC: HPLC analysis was performed on an Agilent series 1100 Liquid Chromatograph and equipped with a quaternary pump system. The mobile phase consists of H_2O (0.05%TFA)/CH_3CN (0.05%TFA)=60/40 (^{V}/v), with a flow rate of 1ml/min. The column used was 4.6mmx250mm. Temperature was 40 ℃ and detection was at 220nm. Their retention times were 7.1min for [(RS)-1] and 6.1min for *(2S, 3S)-2*.

Chiral HPLC: Chiral HPLC analysis was performed on an Agilent series 1050, the column was chiralpak OD and equipped with a quaternary pump system. The mobile phase consists of Heptane/C_2H_5OH=90/10 (^{V}/v), with a flow rate of 1ml/min. The column used was 4.6mmx250mm. Temperature was 30 ℃ and detection was at 250nm. Their retention times were 15.2min for l-hydroxylactam and 24.7min for d-Hydroxylactam.

E. Bioconversion Studies in Bioreator:

The bioconversion studies were carried out in New Brunswick Scientific, USA (NBS) fermenter having a geometric volume of 10L. The fermenter was initially dry sterilized and condensate water was aseptically drained. A 3.6L of 100mM sterile phosphate buffer (pH 6.5) along with the 200g of instant dry yeast was aseptically transferred to the fermenter. The temperature was set at 30 ℃ and agitation at 500rpm. The pH of the reaction was continuously maintained at 6.5 using 5M NaOH in auto-control mode. Dextrose monohydrate (50% solution) was fed to the fermenter with a flow rate of 14.58ml/h from the beginning till 74h and afterwards it was increased to 23.33ml/h till 89h. Towards the end, after 89h the flow rate was maintained with 14.58ml/h till end of the reaction. The 200g of [(RS)-1] was dissolved in 400ml of DMF and added to the reaction mixture in the fermenter at the rate of 9.52ml/h from time zero to 42h. Samples were withdrawn from the fermentor at regular time interval and processed as mentioned in C for analysis. The reaction was continued till 97h.

F. Isolation of (2S,3S)-2 from the Reaction Mixture:

The reaction mixture from the fermenter was centrifuged at 5000rpm for 30min and the supernatant was discarded. The pellet was extracted with 6L of CH_2Cl_2 and then centrifuged at 5000rpm for 30min to get the organic layer. The pellet was further extracted with same volume of CH_2Cl_2 and centrifuged. The organic layer was pooled together and concentrated to dryness using rotary evaporator at 45 ℃. The CH_2Cl_2 extract was refluxed twice in toluene ($C_6H_5CH_3$) and filtered. The filtrate was pooled and chilled to 0-5 ℃ and further filtered to recover the solid product.

III. Results and Discussion

The Diltiazem hydrochloride synthesis starting from p-anisaldehyde is shown in fig 1. In this process, the racemic mixture of acid is formed out of which around 50% contributes to the cis-l-(-) acid, which is a waste product. For the effective utilization of this waste, it was reduced to [(RS)-1] using chemical synthesis, which was then used as a starting substrate for bioconversion to *(2S, 3S)-2* in the present study.

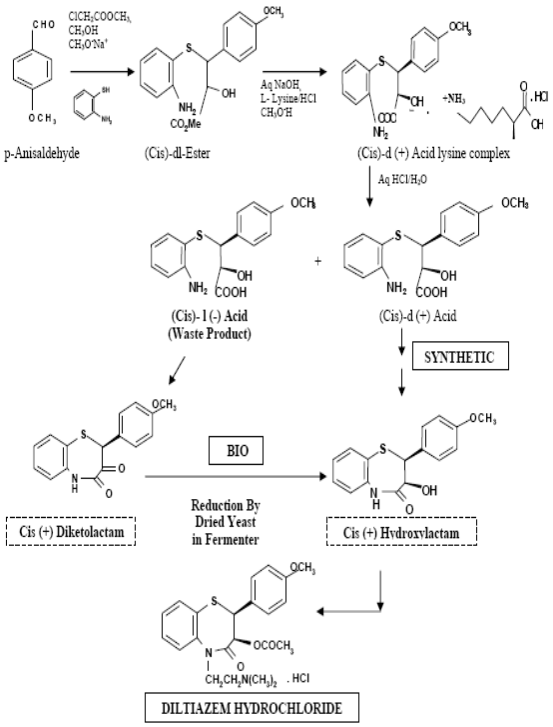

Fig. 1. Synthesis of diltiazem hydrochloride by synthetic and biological route.

Stereo selective reduction of ketone to the desired isomer has been reported. In this paper we report the reduction of [(RS)-1] to the desired isomer *(2S, 3S)-2* using biotransformation catalyzed by whole cells of bakers' yeast. Reports are there where various microorganisms were

screened to carryout the reduction of [(RS)-1] to the desired isomer. They observed that the reduction by various organisms produced mixture of stereoisomer with that *(2S, 3S)-2* stereoisomer being the major product.

The bakers' yeast was found to give highest conversion yield and excellent stereo selectivity for the production of *(2S, 3S)-2* isomer. Diltiazem synthesis through asymmetric reduction by bakers' yeast compares favorably with that through optical resolution by lipase as a synthetic route. In the present work initially we screened different brands of bakers' yeast in shake flask to find outthe best strain for bioconversion. Out of different lots tested, the Meteoric brand dried yeast was found to be the best and hence was used for further studies.

Different trials were taken in the flask to find out the optimum conditions for desired bioconversion. Table (I) describes the conversion obtained with varying conditions like yeast cell concentration, substrate concentration, glucose dosing, pH of the buffer and temperature. The optimum conversions of around with the buffer pH 6.5, temperature 30 ℃ and substrate concentration 50g/L under shake flask condition. Shake flask studies have many limitations as the feed rate cannot be controlled andthe continuous maintenance of pH is very difficult. Hence these conditions were used for scale up studies in laboratory scale fermentor.

The large-scale production of Diltiazem intermediates has been reported a using aggregate of [(RS)-1], which was reduced by bakers' yeast under shake flask cultivation. The author described the difficulty of the bakers' yeast mediated reduction of [(RS)-1] for industrial application due to low substrate concentration.

Hence to improve the product concentration, different procedures of substrate addition was followed like use of aggregates prepared from crystals of [(RS)-1] or DMF soluble substrates.

While using DMF as a vehicle for substrate delivery to the reaction mixture, there is a possibility that large amount of DMF may inactivate yeast cells and thereby inhibiting reduction. Hence a fed batch operation using DMF solution of [(RS)-1] was reported for large scale production of *(2S,3S)-2* at a shake flask level. However there are no reports on this bioconversion at the laboratory scale fermentor level.

In the present study DMF soluble substrate [(RS)-1] was reduced under fed batch conditions using Meteroic bakers' yeast. The DMF soluble substrate was slowly added to the reaction mixture at the rate of 9.52ml/h and the entire 200g substrate addition was completed by 42h.

The slow substrate addition helped in formation of desired product with better conversion efficiency. After complete addition of the substrate, there was an increase in DMF concentration in the reaction mixture, which lowered the conversion efficiency. The continuous maintenance of pH and dextrose dosing helped in maintaining the viability of cells thereby achieving further improvement in bioconversion after 74h. By 89h the maximum conversion of 79.33% was obtained and hence the reaction was terminated by 97h. Fig 2 and 3 shows the HPLC chromatogram of initial substrate [(RS)-1] and converted product *(2S, 3S)-2* respectively. HPLC chromatogram of the sample from the reaction mixture after 2h is shown in fig 4, which indicates the beginning of the bioconversion with the formation of *(2S, 3S)-2*. Towards the end of the reaction at 89h, the maximum conversion of substrate to desired isomer is seen from the HPLC chromatogram from fig 5.

This process could be further optimized to get improvements in bioconversion efficiency. The attempts were made to isolate the crystals of cis (+) hydroxylactam from the reaction mixture. The initial input of the substrate was 200g and with 79.33% of conversion the expected yield of the product was 159g as shown in fig 6. The isolation procedure that was followed could able to yield 66.78g of desired product with 42% of purification efficiency. The separation efficiency could be further improved for achieving higher yields.

TABLE I: SHAKE FLASK TRIALS

Varying Parameters					HPLC (Area%)	
Dried yeast (g/L)	Substrate (g/L)	Dextrose dosed (ml/h)	Buffer pH	Temperature	Hydroxy Lactam	Diketo Lactam
100	75	1.92	6.5	30	15.59	78.67
150	75	1.92	6.5	30	15.9	76.42
200	75	1.92	6.5	30	16.75	73.94
250	75	1.92	6.5	30	16.98	72.5
100	**50**	**1.92**	**6.5**	**30**	**53.77**	**39.44**
100	50	1.92	6.5	25	24.85	75.06
100	50	1.92	5	30	8.71	82.89
100	50	1.92	7.6	30	30.33	62.06
100	50	3.84	6.5	30	17.5	75.32

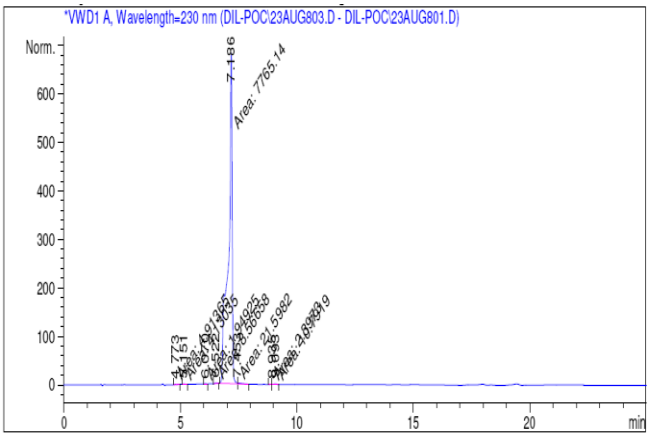

Fig 2. Initial substrate [(RS)-1]

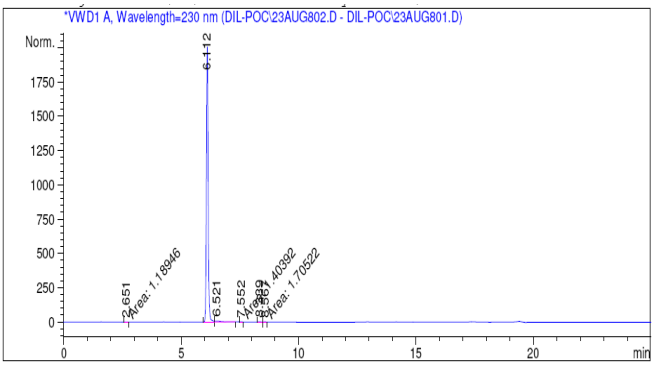

Fig 3. Final product *[(2S, 3S)-2]*

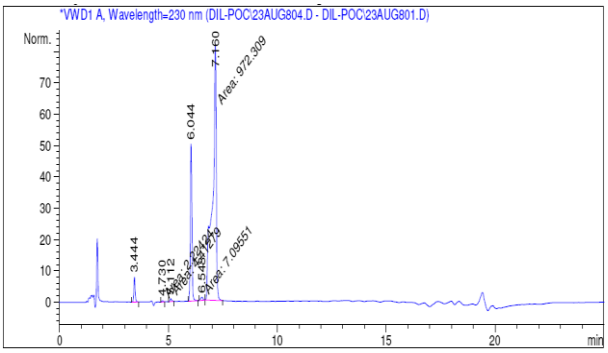

Fig 4. Reaction mixture at 2h

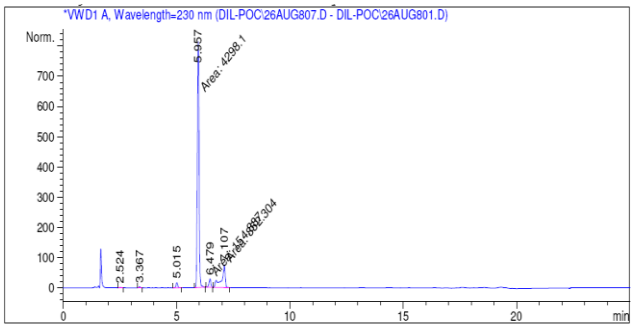

Fig 5. Reaction mixture at 89h

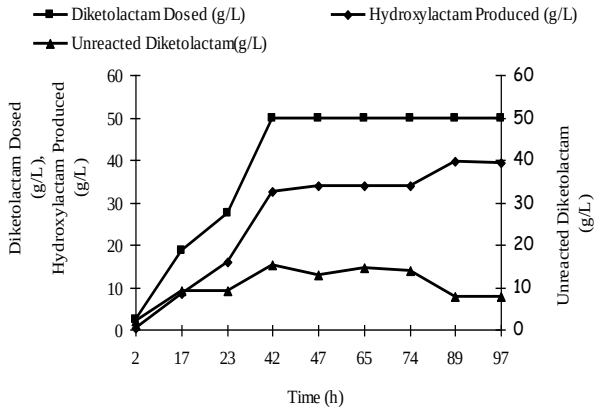

Fig 6. In-process analysis.

IV. Conclusion

The reduction of (*RS*)-2-(4-methoxyphenyl)-l, 5-benzothiazepin-3,4(*2H,5H*)-dione[(*RS*)-1] recovered from the oxidation of alcohol from effluent mother liquor using bakers' yeast was successfully demonstrated in a laboratory scale 10L fermenter. The maximum conversion of 79.33% was observed in this study within 89h of reaction using 200g of substrate. The desired intermediate was isolated from the reaction mixture with 42% of recovery having 95.42% of assay purity with 99.80% of enantiomeric excess.

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