

Optimization of Various Parameters for the Bioconversion of AD to ADD by using *Arthrobacter simplex*

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ABSTRACT: The enhanced bioconversion of 4-androstene-3,17-dione (AD) to Androst-1,4-diene-3,17-dione (ADD) using *Arthrobacter simplex* was demonstrated by optimizing various process parameters such as growth parameters, substrate concentration, inducer concentration and its induction time. The growth parameters for *A. simplex* were optimized at lab level and seed level. The objective of steroid biotransformation is to improve the product formation by optimizing the concentration of substrate addition. The optimal concentration of substrate-10g/l was found to be favourable for increasing bioconversion rate. The biotransformation rate is also enhanced by increasing the 3-Ketosteroid $\Delta(1)$ -dehydrogenase enzymatic activity of culture with the addition of inducer (cortisone acetate 250 ppm) at 05 hrs in grown production medium which is responsible for steroids bioconversion. Thus, this strategy provides a significant way to enhance the bioconversion of AD to ADD.

KEYWORDS: Bioconversion, Androst-1,4-diene-3,17-dione (ADD), 4-androstene-3,17-dione(AD), *Arthrobacter simplex*, process parameter

I. INTRODUCTION

Steroids are compounds possessing the skeleton of cyclopenta- α -phenanthrene. They are natural organic compounds widely distributed in eukaryotic organisms. Steroid drug intermediates are widely used for the commercial production of pharmaceutical steroid drugs such as corticosteroids, mineralocorticoids, oral contraceptives, etc. Among these steroid intermediates, 4-androstene-3,17-dione (AD) and androst-1,4-diene-3,17-dione (ADD) are the two major products usually used as the starting material to prepare various kinds of important pharmaceutical steroids [1].

Microbial steroid transformation which exploits the metabolic and biocatalytic potential of the microorganisms, is a powerful tool for generation of novel steroidal drugs, as well as their intermediates. Since the discovery that microorganisms could degrade the side-chain of sterols to produce C17-ketosteroids [2]. The technique has many reaction-specific processes; higher stereospecificity; milder reaction condition and are therefore preferred over chemical transformation. Many microbial strains have been reported capable of converting sterols to ADD including *Aspergillus*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Chryseobacterium*, *Fusarium*, *Gordonia*, *Nocardia*, *Pseudomonas*, *Rhodococcus* and *Streptomyces* [3, 4].

Arthrobacter simplex is a gram-positive bacterium showing a rod-coccus growth cycle. It has been extensively used in pharmaceutical industry to introduce C1-C2 double bond into steroids. 3-ketosteroid- $\Delta(1)$ -dehydrogenase (KSDH) of the species is the key enzyme in bioconversion to androstadienedione. 3-Ketosteroid- $\Delta(1)$ -dehydrogenase from *A. simplex* catalyzes transhydrogenation of 3-keto-4-ene-steroid to 3-keto-1,4-diene-steroid e.g., 4-androstene-3,17-dione

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

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to 1,4-androstadiene-3,17-dione. This FAD-dependent enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-CH group of donor with other acceptors. The *A. simplex* cells are induced for steroid-1-dehydrogenase activity by the addition of a 1,2-saturated 3-keto-steroid compound such as cortisone acetate. Cortisone acetate is a glucocorticoid, a white to practically white, odorless, crystalline powder. The inducer can be added at any point during the growth cycle.

In several biotransformation processes, both substrates and products have poor aqueous solubility, thereby decreasing the transport rates to and from the cells, resulting in low yields of the products [5,6]. 4-androstene-3,17-dione (AD) is practically insoluble in water, soluble in solvents as alcohol, acetone or dioxane. Cyclodextrins have been widely known to increase the aqueous solubility of various lipophilic substrates. Cyclodextrins (CDs) are cyclic oligosaccharides with 6–8 glucopyranose units bonded by α -1,4- linkage and have a structure that conveys a hydrophobic central cavity in the molecule [7]. Based on this molecular structure, cyclodextrin have been widely used to form host-guest complexes with hydrophobic compounds in their cavities. By inclusion with cyclodextrin, the hydrophobic compounds are encapsulated in this cavity. Hydroxypropyl- β -cyclodextrin (HP- β -CD) is one kind of chemically modified β -cyclodextrin derivate, which has higher water solubility and complexing ability than cyclodextrin, and has been widely added in medium to improve steroid biotransformation [8].

The present study is a novel approach that describes the improved bioconversion of 4-androstene-3,17-dione (AD) to androst-1,4-diene-3,17-dione (ADD) using *A. simplex*, a key intermediate for the production of steroidal drugs and hormones. In the present study, the various parameters were studied like growth parameters, inducer concentration and its induction time, substrate concentration was studied.

II. MATERIAL AND METHODS

Microorganism:

In this study, *A. simplex* ATCC 6946 was used for the conversion of AD to ADD. The culture was maintained on Nutrient Agar media at 4°C.

Bioconversion using *A. simplex* ATCC 6946

Growth in lab media and seed media:

The culture was used to inoculate in lab media (50 ml media in 250ml Erlenmeyer flasks) containing Dipotassium hydrogen phosphate- 4.0 g/l, Potassium dihydrogen phosphate- 2.0 g/l, Magnesium sulphate- 0.50 g/l, Yeast extract- 6.0 g/l, Soya peptone- 6.0 g/l, Dextrose- 20 g/l and pH- 7.0. The culture was incubated in orbital shaking incubator at 30°C and 240rpm.

The seed media was prepared in 250ml Erlenmeyer flask (Dipotassium hydrogen phosphate- 4.0 g/l, Potassium dihydrogen phosphate- 2.0 g/l, Magnesium sulphate- 0.50 g/l, Yeast extract- 6.0 g/l, Soya peptone- 6.0 g/l, Dextrose- 20 g/l and pH- 7.0). 0.5% grown lab was used to inoculate seed flask. Incubation was done in orbital shaking incubator at 30°C and 240rpm for overnight. pH and OD^{600nm} were taken at every 2 hrs interval to know the growth profile or curve of lab and seed culture.

Growth of *A. simplex* in production media:

The production media (50ml in 250ml Erlenmeyer flask) containing Dipotassium hydrogen phosphate- 2.5 g/l, Potassium dihydrogen phosphate- 0.6 g/l, Magnesium sulphate- 0.5 g/l, Yeast extract- 10 g/l, Soya peptone- 10 g/l, Dextrose- 20 g/l and pH 7.0 was inoculated with grown seed culture and incubated at 30°C and 180rpm in orbital shaking incubator for 24hrs.

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Bioconversion of AD to ADD:

The production media was inoculated with 20 hrs mature seed culture and incubated in orbital shaking incubator at 30°C and 180rpm for 20hrs. Induction of cortisone acetate was done at 5 hrs. in production culture. Cells of grown production broth were pellet down by centrifugation at 5000rpm. The cell mass were collected and incorporated in buffer solution, then loaded with substrate buffer .

Estimation of Bioconversion by HPLC method:

Androst-1,4-diene-3,17-dione (ADD) produced in the culture broth was determined by HPLC. The culture broth of 2.5 gm was taken in 25 ml volumetric flask with 10 ml methanol and sonicated for 20 minutes. The extract was filtered and diluted with methanol and injected in the system. The HPLC (Waters 2496) having C-18 column (Betasil) was used for the estimation of ADD at 238nm. The mobile phase was composed of Water:Acetonitrile, the flow rate was 0.8 ml/min and column temperature at 30°C. Concentration of ADD was calculated by comparison of peak areas with the standard ADD and subsequently ADD activity was calculated.

Enhancement of bioconversion for ADD:

Following parameters were selected to maximize the bioconversion for the production of ADD.

A: Impact of Inducer on growth profile:

Induction of cortisone acetate was done in production culture. Cortisone acetate play the role of inducer. It induces the enzyme 3-Ketosteroid $\Delta(1)$ -dehydrogenases, responsible for conversion of AD to ADD. The enzyme catalyzed the conversion of several 3-keto-steroids. To know the impact of inducer, 2 set of experiments were designed (Table 1). To obtain the data, growth parameters were analyzed at every 2 hrs interval.

Table 1: 2 Set of Experiments

Experiment	Inducer
1	+
2	-

B: Impact of Induction of different interval:

The cells of *A. simplex* were induced for enzyme steroid-1-dehydrogenase activity by the addition of cortisone acetate (1,2-saturated 3-keto-steroid compound). After knowing the impact of inducer, it is necessary to know the induction time of cortisone acetate in production culture. 4 sets of experiments were planned to target the age of 3hrs., 4hrs, 5hrs. and 6hrs. respectively to feed the inducer for the betterment of bioconversion.

C: Impact of Inducer with different Concentration:

On the basis of above knowledge, it is important to focus on the concentration of inducer to be fed. The concentration of cortisone acetate was 50 ppm to 500 ppm viz. 50, 100, 150, 200, 250, 300, 350, 400, 450 and 500ppm respectively. The feeding was done at 5 hrs of production culture. The experiment was repeated in triplicate.

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D:Variation in Substrate concentration:

It is required to investigate the impact of increasing the AD concentration on bioconversion. To maximize the production of ADD, substrate concentration was increased from 10-20g/l i.e., 10, 15, 20 and 25g/l respectively. The cyclodextrin was dissolved in distilled water at 70°C for an hour. Substrate was added gradually in saturated cyclodextrin solution with continuous agitation till the complete homogenization before sterilization.

Incorporation of cells in Buffer Solution and addition of substrate for conversion:

The grown production broth was pellet down by centrifugation at 5000rpm. The collected cell mass was incorporated in buffer solution (Di-sodium hydrogen phosphate- 3.0g/l, Sodium di-hydrogen phosphate- 0.1g/l) and the flasks were allowed to shake for homogenous distribution of cells in this solution. After uniform distribution of cells, substrate (slurry) was added. The flasks were incubated in shaker at 26°C and 180rpm for the bioconversion of AD to ADD.

III. RESULT AND DISCUSSION

Enrichment of culture is most important. It is necessary to provide carbon sources, nitrogen sources, minerals, salts for osmotic balance of culture and required water for enrichment. In lab media, the culture is enriched with required nutrients. To increase the purity of culture and minimize the toxicity of the medium, cells are nourished in lab media. After activation of culture, it is important to increase the inoculum size for the fermentation process. The lab culture was then activated in seed medium. Seed medium is required for the increment of inoculum size and minimize the lethal effect of cells.

pH and OD was taken at every 2 hrs. interval to know the concentration of cells in medium.

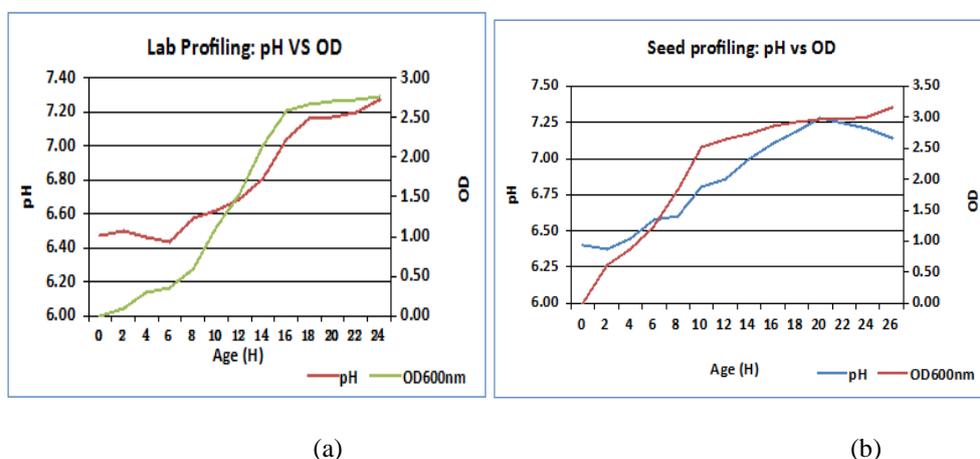


Fig.1: Growth curve for (a) lab and (b) seed culture.

Fig. 1 illustrated the optimum age for lab (a) and seed (b) culture was 20 hrs with. The optimized pH 7.17 and OD 2.709 for lab culture and pH 7.28 and OD 2.961 at 20 hrs seed inoculum. pH was in down trend or acidic and concentration of cells were stable or constant at this age.

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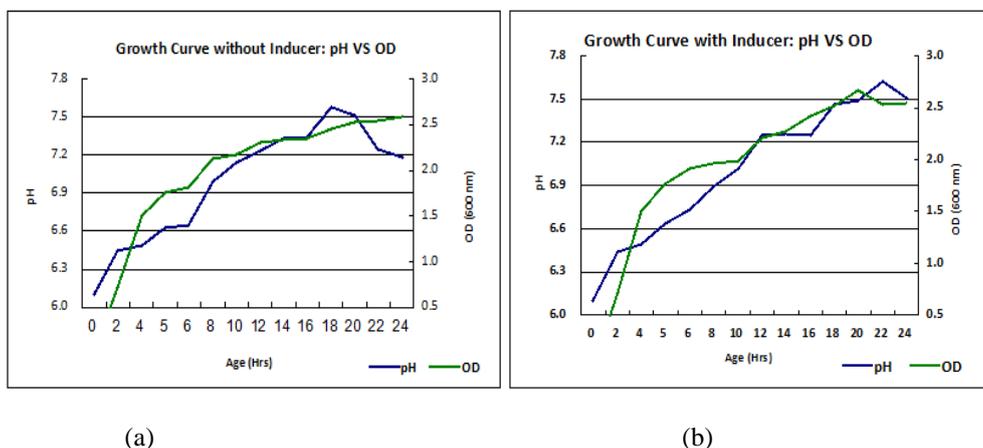


Fig.2: Impact of Inducer on growth

Fig. 2 is demonstrated that cell density is increased by the induction of cortisone acetate. Cortisone acetate play the role to enhance the enzymatic activity of of the culture which is directly correlated with the concentration of cells. This enzyme catalyzes transhydrogenation of 3-keto-4-ene-steroid to 3-keto-1,4-diene-steroid.

Table 2: Age for induction of inducer

Age of production culture (hr)	% bioconversion
3	20.17
4	29.26
5	33.33
6	24.76

Maximum bioconversion of ADD was observed by induction of cortisone acetate at 5 hrs, in production culture (Table 2). The enzyme activity was increased by inducing of cortisone acetate at 5 hrs. After this age the effect of enzymatic activity was less. However, the early feeding of cortisone acetate in the production culture was not induces the dehydrogenase activity of enzyme as per the requirement for the steroid metabolism.

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Table 3: Concentration of inducer

Inducer concentration (ppm)	% bioconversion
50	29.13
100	34.62
150	38.99
200	42.68
250	50.99
300	46.69
350	42.11
400	31.72
450	25.21
500	12.82

Table 3 manifests that the maximum bioconversion *i.e.*, ~50% was observed by feeding of 250 ppm cortisone acetate. The bioconversion rate is enhanced by increasing the enzymatic activity of culture. The enzyme production ability of the culture is insufficient to excrete the overproduction of 3-Ketosteroid $\Delta(1)$ -dehydrogenase. Overproduction of the enzyme, 3-Ketosteroid $\Delta(1)$ -dehydrogenase, is necessary for the betterment of bioconversion. The toxicity in the medium was increased by increasing the concentration of inducer.

Table 4: Effect of substrate concentration

Substrate concentration (g/l)	ADD formation	% bioconversion
10	8.12	81.20
15	10.68	71.20
20	13.24	66.20
25	14.22	56.88

Table 4 showed that ~80% bioconversion was observed by loading 10g/l AD. Steroid bioconversion have been reported to be affected by substrate inhibition [10]. The objective of steroid bioconversion is to enhance the product formation with high substrate addition. It is clearly visualized (Table 4) that the product accumulation was not accelerated with

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the increased concentration of substrate. It is important to focus on the solubility of substrate. Low solubility of substrate decreases the bioconversion rate of steroids. The solubility of substrate decreases as the concentration of substrate increases. With increasing the concentration of substrate, the product accumulation (ADD) was maximized. But its conversion rate decreased due to the low substrate solubility. The main focus of steroid biotransformation is to increase the solubility of hydrophobic substrates as well as retain the activity and stability of microbial cells. Cyclodextrins have been widely known to increase the aqueous solubility of various lipophilic substrates. Cyclodextrin plays a significant role in bioconversion of steroids. Cyclodextrins (CDs) are cyclic oligosaccharides with 6–8 glucopyranose units bonded by α -1,4-linkage and have a structure that conveys a hydrophobic central cavity in the molecule [7]. It retains a significant solubility and complexing ability and has been widely added in medium to improve steroid biotransformation [8, 9, 11]. It increases the aqueous solubilization of steroid molecules. The cyclodextrin molecule has a torus shape, with the hydrophilic portion outside and hydrophobic inside the cavity. Substrate molecule is held within the cavity of cyclodextrin and form the molecular complexation (Substrate- β CD inclusion complex). The main focus of steroid biotransformation is to increase the solubility of hydrophobic substrates as well as retain the activity and stability of microbial cells.

IV. CONCLUSION

The objective of this study was to enhance the bioconversion rate for the product formation by change in substrate concentration in such a way that it is easily available to cell for conversion. The current study showed that the aqueous solubility of substrate can lead to the increment of bioconversion. The optimal concentration of substrate was 10g/l that showed greater solubility. Furthermore, the impact of enzymatic activity of culture on the bioconversion rate was studied. The incorporation of inducer (cortisone acetate 250ppm) at 5 hrs in production culture increased the dehydrogenase activity, responsible for steroids metabolism. In present study, the bioconversion rate of 4-androstene-3,17-dione (AD) to androst-1,4-diene-3,17-dione (ADD) using *Arthrobacter simplex* was increased to manifolds.

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