



Journal of Applied Sciences

ISSN 1812-5654

science
alert

ANSI*net*
an open access publisher
<http://ansinet.com>

Effect of pH on the Biotransformation of (*R*)-1-(4-bromo-phenyl)-ethanol by using *Aspergillus niger* as Biocatalyst

F.Z. Abas, M.H. Uzir and M.H.M. Zahar

School of Chemical Engineering, Engineering Campus, Universiti Sains Malaysia,
Seri Ampangan, 14300 Nibong Tebal, S.P.S Penang, Malaysia

Abstract: Molecular chirality is a fundamental phenomenon that plays an important role in biological processes. Currently, much attention has been focused on the production of chiral alcohols. Chiral alcohols such as (*R*)-1-(4-bromo-phenyl)-ethanol is important as a useful starting material used in pharmaceutical industries for drug synthesis. There are many factors that influenced the bioconversion and some of the crucial ones include the pH of media, biotransformation time, substrate concentration and agitation speed. All of these factors significantly affect the percentage of enantiomeric excess as well as the percentage conversion of the chiral alcohol. In this work, biocatalytic production of (*R*)-1-(4-bromo-phenyl)-ethanol was achieved via asymmetric reduction of 1-(4-bromo-phenyl)-ethanone using the shake-flask method at the reaction conditions of 30° C, 1 mmol of substrate, agitation speed of 150 rpm and at various pH's. Since *Aspergillus niger* can easily grow and produce enzyme that is suitable in converting the substrate, therefore, it was used as a biocatalyst in the reaction. Based on the results obtained, pH 7 gave the highest enantiomeric excess of 99.9% and conversion of 94.7% for the reduction time of 48 h.

Key words: Chiral alcohol, (*R*)-1-(4-bromo-phenyl)-ethanol, pH, *Aspergillus niger*, fermentation, biocatalyst, bioreduction

INTRODUCTION

Biotransformation process has been increasingly utilized in order to replace the conventional chemical processes and to facilitate the formation of new products in many areas such as pharmaceutical and agrochemical to mention a few (Goretti *et al.*, 2009). The technique is now established as a useful tool in the production of intermediates in fine chemical processes (Valadez-Blanco and Livingston, 2009). One of the most successful and popular applications in biotransformation is the asymmetric reduction of substituent of acetophenone to a chiral phenylethanol using the whole-cell biocatalyst. According to Xiao *et al.* (2005) biotransformation using growing cells is still a preferable method for the synthesis of most of the cofactor-dependent products industrially. Valadez-Blanco and Livingston (2009) reported that the enantioselective reduction of ketones in organic synthesis plays a major role in the production of chiral intermediates. The reaction of acetophenone to chiral phenylethanol has also been widely studied as a model reaction for ketone bioreductions in order to produce a specific chiral alcohol.

Chirality is a key factor in the efficiency of many enantiomers (Zilbeyaz and Kurbanoglu, 2008).

Enantiomerically pure secondary chiral alcohols such as (*R*)-1-(4-bromo-phenyl)-ethanol as well as a chiral alcohol with additional functional group serve as an important chiral building block and a useful starting materials for the synthesis of various biologically active compound especially in pharmaceutical, chemical and agrochemicals industries (Shimizu *et al.*, 1998; Zilbeyaz *et al.*, 2010). In fact, the demand for chiral alcohols has recently boosted in accordance to the need for optically active drugs (Kurbanoglu *et al.*, 2007). These chiral alcohols have also been utilized as ligands for various metals for a number of asymmetric reactions (Moon Kim and Jin Kyoony, 1999).

This study is aimed to achieve in particular the highest product's enantiomeric excess as well as the conversion by verifying the pH of reaction medium from 6 to 9. The model reaction is the asymmetric reduction of 1-(4-bromo-phenyl)-ethanone by *Aspergillus niger* producing (*R*)-1-(4-bromo-phenyl)-ethanol.

BIOCATALYST FOR THE SYNTHESIS OF CHIRAL ALCOHOL

Whole-cells microorganisms are normally preferred in many biocatalytic applications. Jurcek *et al.* (2008) reported that there are increasingly important of using

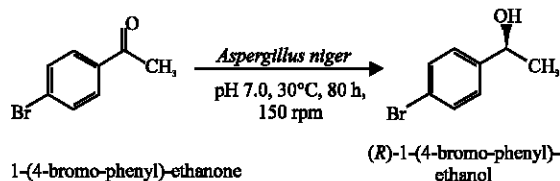


Fig. 1: Asymmetric reduction of 1-(4-bromo-phenyl)-ethanone to (R)-1-(4-bromo-phenyl) ethanol by using *A. niger* (Zilbeyaz and Kurbanoglu, 2008)

microorganisms for relatively easy introduction of chiral centers into the new molecules. This has been based on the fact that microorganisms are able to transform a great variety of organic molecules, both natural and synthetic, into the requested chiral products with high enantioselectivity (Jurcek *et al.*, 2008). The enantiopure alcohols which are significantly employed as chiral building blocks for fine chemicals can be prepared through the biocatalytic methods using the whole-cell microorganisms as biocatalyst (Drepper *et al.*, 2006).

Figure 1 shows the schematic process of reduction of 1-(4-bromo-phenyl)-ethanone to (R)-1-(4-Bromo-phenyl)-ethanol using *Aspergillus niger* as a biocatalyst at a specified condition.

Moreover, Zilbeyaz and Kurbanoglu (2008) found that the whole-cell of *A. niger* fungus is an effective biocatalyst for this enantioselective bioreduction to obtain the corresponding (R)-alcohol. Therefore, as the biocatalytic reaction are carried out by the whole fungal cells, it was observed that the ketone was reduced to its corresponding alcohol, which means that the alcohol dehydrogenases is present in the enzymatic system of the microorganisms (Keppler *et al.*, 2005). The usage of *A. niger* is significantly effective since the application of whole-cell is rather simple, generally cheaper and highly advantageous for practical synthesis of chiral alcohols (Shimizu *et al.*, 1998). In addition, the usage of whole microbial cells is preferable for carrying out the desired reduction process since they do not require the addition of NADPH/NADP cofactors for regeneration. This is due to the fact that they contain multiple dehydrogenases and all the enzymes as well as cofactors are well protected within their natural cellular environment (Mandal *et al.*, 2004). As a result, the enzyme becomes more stable and thus, extending the life of a biocatalyst (Zilbeyaz and Kurbanoglu, 2008). Likewise, it properly acts under mild reaction conditions, biodegradable and environmentally friendly and has a remarkable predominance due to their high enantioselectivity (Xiao *et al.*, 2005). Therefore, an increasing important application of microorganism for relatively easy

introduction of chiral centers into the new molecules has been mainly based on the fact that microorganisms are able to transform a great variety of organic molecules, both natural and synthetic, into the desired chiral products with high enantioselectivity

COMPARISON BETWEEN BIOLOGICAL AND CHEMICAL REACTIONS

According to Nakamura *et al.* (2003) biocatalysis has a unique characteristic when compared to the chemical catalyst. Some features that distinguish biocatalyst from chemical catalyst are listed as follow:

- **Catalyst preparation:** Some of the biocatalysts for asymmetric reduction, such as the isolated-enzyme and whole-cells are commercially available and ready to use
- **Selectivity:** Very high enantio-, regio- and chemo-selectivities can be achieved due to the strict recognition of the substrate by enzymes. For example, in the reduction of ethyl propyl ketone, biological based reaction has achieved high enantioselectivities (98%). In contrast, chemical catalyst can perform highly enantioselective reduction when two adjacent groups of the carbonyl carbon of the ketones are significantly different
- **Safety of the reaction:** Biocatalytic reductions are generally safe. The reaction conditions are mild, the solvent is usually water and hazardous reagents are not necessary. For instance, ethanol and glucose were used as hydrogen sources instead of explosive hydrogen gas. This is because hydrogen sources are necessary to perform a reduction reaction
- **Natural catalysts:** Biocatalysts, i.e., microorganisms, plants, animal cells, or their isolated enzymes, are reproducible and can be easily decomposed in the environment after use

Nowadays, there is a considerable interest in finding the most efficient routes to produce chiral alcohols. The compound can be synthesized in enantiomerically pure form from prochiral ketones either biologically, using a biocatalytic system, or chemically via stereoselective reduction, using either a catalytic system or a stoichiometric amount of reducing agent (Hage *et al.*, 2001). Both the fungus (*Mellierius tremellosus*) and the metal based catalysts (iridium) are suitable systems for aryl ketone reduction but there is a slight difference on the percentage of Enantiomeric Excess (% e.e) and product yield/conversion gained. The results are summarized as in Table 1.

Table 1: Alcohol production from biocatalyzed and metal-catalyzed asymmetric reductions of aryl ketones

Ketone	<i>M. tremellosus</i> (%)		Iridium (%)	
	Yield	ee	Conv.	ee
1	61	95	82	80 (R)
2	53	96	Nd	Nd
3	88	97	Nd	Nd
4	Nd	Nd	95	92 (R)
5	49	98	Nd	Nd
6	Nd	Nd	99	77 (R)
7	56	98	52	88 (R)
8	41	90	97	97 (R)
9	63	Rac.	97	87 (R)
10	68	95	39	37 (R)

Nd: Not defined, Rac: Racemic, ee: Enantiomeric excess

According to Table 1, the comparison of results showed that the corresponding chiral alcohols could be obtained with moderate to high enantioselectivities (e.e.'s up to 98%). In addition, it also shows that biocatalytic approach using the fungus is the most suitable for the enantioselective reduction of aryl ketones.

MATERIALS AND METHODS

Culture medium and biotransformation: The method is a modification from the study of Zilbeyaz and Kurbanoglu (2008). The media fermentation was prepared with addition of glucose 20 g L⁻¹, yeast extract 3 g L⁻¹ and normal peptone 4 g L⁻¹ in the 1 L of Erlenmeyer flask. Then, before it was sterilized at 121°C for 15 min, the culture medium was set at pH 7 using 1 N HCl and 1 N NaOH. The pH of the culture medium was then adjusted to 6.0, 6.5, 7.5, 8.0, 8.5 and 9.0 with the same molar of HCl and NaOH. The pH of the media was kept constant throughout 80 h biotransformation time. All cultures were grown in 250 mL of the Erlenmeyer flasks containing 150 mL of culture medium. The conidia from 5 days old cultures that were already prepared were used for inoculation. The conidial suspension was prepared in a sterilized 200 mL distilled water by gently scratching the conidia from an agar plate with a sterile wire loop and then vigorously shaken to break the conidial clumps. About 15 mL of conidial suspension was then added into each flask. The organism was maintained at the same weight for all flasks, i.e., 5 g L⁻¹. Flasks were incubated in an orbital shaker (Labtech LSI-3016 A) at 30°C and 150 rpm. Only after 48 h of fermentation time, 1-(4-bromo-phenyl)-ethanone (1 mmol) was added directly to each culture medium and then the incubation was continued at 30°C and 150 rpm for 60 h reduction time (Zilbeyaz and Kurbanoglu, 2008).

Purification of product: After 4 h of incubation, the first sample from each pH was withdrawn for purification and analysis. The samples were withdrawn in every 4 h as the

reduction progressed. The mycelium was separated by filtration and the filtrate was saturated with sodium chloride and then extracted with ethyl acetate. The mycelia were also extracted using the same solvent. The extracts were combined and then dried over Na₂SO₄ to remove any moisture content.

Analytical method: The consideration of analytical methodologies needed for the determination of chiral compounds in pharmaceutical and biological samples is a key component to the successful compound of chiral drugs (Williams *et al.*, 1998). For this process, 2 mL of the extracted product which was that previously purified was centrifuged in order to separate the two phase of product sample using microcentrifuge (Profuge 6K) at 6600 rpm for 10 min. The upper layer of the product which is organic solvent was withdrawn using the syringe and was put into 10 mL of beaker. After that, the product sample was filtered using the 0.45 µm nylon membrane filter (Double Rings Filter Paper, 101) before it was put into the bottle sample. Then the samples were injected into the HPLC. HPLC with Regis Reversible HPLC OD Column (4.6×250 mm, 10 µm) purchased from Fisher Scientific using the eluent of n-hexane-i-Propanol, 90:10, with flow rate of 1.0 mL min⁻¹ and detections was performed at 254 nm at 1 mL min⁻¹ of flow rate. All experiments were replicated twice and averaged values were presented in this study. The optical purity was expressed by the e.e value as given by:

$$\text{Enantiomeric excess, e.e (\%)} = \frac{[R-S]}{[R+S]} \times 100 \quad (1)$$

Meanwhile, the conversion rate was determined from the ratio of reacted substrate concentration, ([S₀]-[S_t]) to its initial substrate concentration ([S₀]) given by:

$$\text{Conversion (\%)} = \frac{([S_0]-[S_t])}{([S_0])} \times 100 \quad (2)$$

RESULTS AND DISCUSSION

pH profiles in the range between 6.0-9.0 were examined for the synthesis of (R)-1-(4-bromo-phenyl)-ethanol. The initial substrate concentration for this experiment was 1 mmol and the initial pH was set at 7. These ranges of pH were analyzed for the optimization of microbial transformation process so that it can be utilized for further analysis. It is well known that pH plays a crucial role in any enzymatic reaction. As can be recognized, variation in pH will alter the ionic state of substrate and the enzymes involved in this reaction thus,

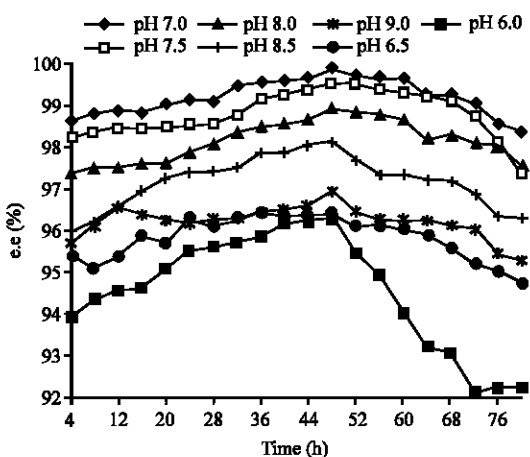


Fig. 2: Enantiomeric excess profile on different pH. Initial substrate concentration 1 mM, agitation 150 rpm, temperature 30°C

leading to the change in enzymatic activities and enantioselectivity (Lou *et al.*, 2004). Figure 2 shows the profile of the enantiomeric excess performance for differential pH.

Based on Fig. 2, it is apparent that the highest enantiomeric excess was achieved at pH 7. The percentage of enantiomeric excess maintained around 98 to 99%. The best reduction time to carry out the synthesis was found at 48 h reduction time with 99.9% enantiomeric excess. After 48 h reduction time, it decreased slightly to 98.3% at 80 h reduction time. Meanwhile, for pH 6.0, it has a weak biotransformation which gave the lowest percentage of enantiomeric excess (92.2%) at 80 h reduction time. The lower enantioselectivity at lower pH could be due to the activity of the enzyme and normally the relationship between the bacterial growth rate and pH is approximately parabolic where when it close to optimum pH value, the growth rate changes little with changes in extracellular pH but it declines more rapidly close to the acidic and alkaline growth limits (Krist *et al.*, 1998). Moreover, from the figure, all the profiles showed an increment of enantiomeric excess from 4 h to 48 h reduction times but then decrease slightly until 80 h reduction time except for pH 6 which gradually decreases after 48 h reduction process. These results strongly suggested that the variation of the enantioselectivity with pH was directly related to the OH group which is chiral alcohol that produced from reduction of ketone (Wang and Shi, 1998).

Figure 3 shows the comparison of product conversion at different pH. The graph shows that the product conversion for all the pH's increased with time. The product conversion gradually increases as the pH

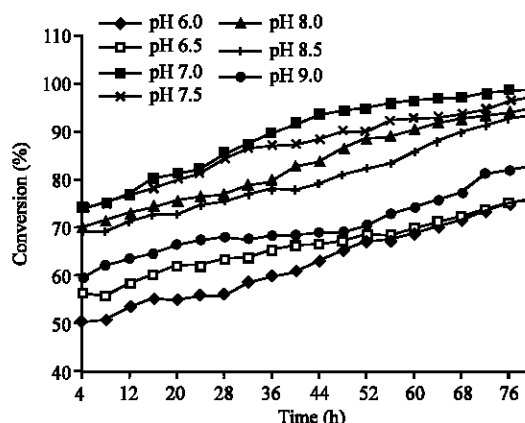


Fig. 3: Conversion profile on for different pH along 80 h incubation time. Initial substrate concentration 1 mM, agitation 150 rpm, temperature 30°C

Table 2: Results of (%) enantiomeric excess and (%) conversion

pH	e.e (%)	Conversion (%)
6.0	96.36	65.6
6.5	96.43	67.6
7.0	99.90	94.7
7.5	99.56	90.2
8.0	98.96	86.8
8.5	98.16	81.2
9.0	96.98	69.2

Initial substrate concentration 1 mmol, 150 rpm, 30°C at reduction time of 48 h

increases. The effect was more apparent with the lower pH. This can be proven when the product conversion gradually decreased when pH was adjusted to 6. Based on the figure, pH 7 gives a good conversion with the highest conversion of 99.3%. Meanwhile, it gives the lowest product conversion at pH 6 as well as pH 6.5 for the period of 80 h. This is because the activity of the enzyme that produces by wild-type of *A. niger* was slow in basic condition. Besides, since the enzymes are protein, they are very sensitive to the changes of pH's and the changes of pH's might affect the shape of an enzyme as well as change the shape or charge properties of the substrate. In addition, generally the activity of enzyme was completely loss at the lowest and highest p (Krist *et al.*, 1998). All these factors will affect the reaction of this reduction as well as the product conversion.

Table 2 shows the results of the percentage of enantiomeric excess and conversion for different pH's at 48 h biotransformation time.

According to Table 1, it clearly shows that the pH significantly influenced the enzymatic enantioselectivity as well as slightly affected the conversion of 1-(4-bromophenyl)-ethanone to form chiral alcohol. The conversion gradually increased when the pH of the growth medium

was increased from pH 6.0 to pH 7. The highest conversion was achieved at 94.7% for pH 7 and it gradually decreased from pH 7.5 to pH 9. Meanwhile the maximum e.e reached 99.9% at pH 7 and drastically decreased to 96.98% at pH 9. Meanwhile, the results give the lowest enantiomeric excess and conversion was achieved at 96.36 and 65.6%, respectively for pH 6. This data was collected at 48 h reduction time. Therefore, based on the graph above, it can be proven that the optimum pH for reduction activity was found to be at pH 7 since it gave the highest e.e as well as highest conversion. Besides, it can be concluded that *Aspergillus niger* has a weak growth as well as biotransformation at pH 6. This is due to the same reason as stated previously in the enantiomeric excess discussion. This is also thought to be due to the homeostatic mechanisms that control the intracellular pH (Krist *et al.*, 1998). In addition, according to the Table 2, comparison of the results show that the corresponding chiral alcohols could be obtained with moderate to high enantioselectivities (e.e up to 100%).

CONCLUSION

(*R*)-1-(4-bromo-phenyl)-ethanol can be effectively produced by the fermentation of the whole-cell *Aspergillus niger* fungus. In this study, the conversion and product e.e were remarkably improves by adjusting the seven different pH levels that can be seen through out the reduction time. Hence, the apparent activity of different enzymes inside the cell maybe regulated through the adjustment of the operation conditions. Among the controllable operating factors such as temperature and concentration of substrate adjustment, pH is the most important factor (Chen *et al.*, 2002). Based on the results obtained, it can be concluded that pH 7 gave the highest e.e which is 99.9% and highest conversion which is 94.7% at 48 h reduction time. This optimum condition can be utilized for further experiment in the future such as for different substrate concentration and different biotransformation time in order to produce the highest percentage of enantioselectivity and product concentration.

ACKNOWLEDGMENTS

Authors would like to thank MOSTI for providing the Science Fund (305/ PJKIMIA/6013204) to carry out this project. F.Z. Abas would also like to thank USM for providing the Fellowship Scholarship under the Research University Scheme.

REFERENCES

- Chen, J., K.P. Wang, J.Y. Houg and S.L. Lee, 2002. Design of the pH profile for asymmetric bioreduction of ethyl 4-chloro-3-oxobutyrate on the basis of a data-driven method. *Biotechnol. Prog.*, 18: 1414-1422.
- Drepper, T., T. Eggert, W. Hummel, C. Leggewie and M. Pohl *et al.*, 2006. Novel biocatalysts for white biotechnology. *Biotechnol. J.*, 1: 777-786.
- Goretti, M., C. Ponzoni, E. Caselli, E. Marchigiani and M.R. Cramarossa *et al.*, 2009. Biotransformation of electron-poor alkenes by yeasts: Asymmetric reduction of (4*S*)-(+)-carvone by yeast enoate reductases. *Enzyme Microbial. Technol.*, 45: 463-468.
- Hage, A., D.G.I. Petra, J.A. Field, D. Schipper and J.B.P.A. Wijnberg *et al.*, 2001. Asymmetric reduction of ketones via whole cell bioconversions and transfer hydrogenation: complementary approaches. *Tetrahedron Asymmetry*, 12: 1025-1034.
- Jurcek, O., M. Wimmerová and Z. Wimmer, 2008. Selected chiral alcohols: Enzymic resolution and reduction of convenient substrates. *Coordination Chem. Rev.*, 252: 767-781.
- Kepler, A.F., A.L.M. Porto, I.H. Schoenlein-Crusius, J.V. Comasseto and L.H. Andrade, 2005. Enzymatic evaluation of different *Aspergillus* strains by biotransformation of cyclic ketones. *Enzyme Microbial. Technol.*, 36: 967-975.
- Krist, K.A., T. Ross and T.A. McMeekin, 1998. Final optical density and growth rate; effects of temperature and NaCl differ from acidity. *Int. J. Food Microbiol.*, 43: 195-203.
- Kurbanoglu, E.B., K. Zilbeyaz, N.I. Kurbanoglu and H. Kilic, 2007. Enantioselective reduction of substituted acetophenones by *Aspergillus niger*. *Tetrahedron Asymmetry*, 18: 1159-1162.
- Lou, W.Y., M.H. Zong, Y.Y. Zhang and H. Wu, 2004. Efficient synthesis of optically active organosilyl alcohol via asymmetric reduction of acyl silane with immobilized yeast. *Enzyme Microbial. Technol.*, 35: 190-196.
- Mandal, D., A. Ahmad, M. Islam Khan and R. Kumar, 2004. Enantioselective bioreduction of acetophenone and its analogous by the fungus *Trichothecium* sp. *J. Mol. Catalysis B Enzymatic*, 27: 61-63.
- Moon Kim, B. and P. Jin Kyoon, 1999. A short synthesis of a chiral alcohol as a new chiral auxiliary for asymmetric reactions. *Bull. Korean Chem. Soc.*, 20: 744-745.
- Nakamura, K., R. Yamanaka, T. Matsuda and T. Harada, 2003. Recent developments in asymmetric reduction of ketones with biocatalysts. *Tetrahedron Asymmetry*, 14: 2659-2681.

- Shimizu, S., M. Kataoka and K. Kita, 1998. Chiral alcohol synthesis with yeast carbonyl reductases. *J. Mol. Catalysis B Enzymatic*, 5: 321-325.
- Valadez-Blanco, R. and A.G. Livingston, 2009. Enantioselective whole-cell biotransformation of acetophenone to S-phenylethanol by *Rhodotorula glutinis*: Part I. Product formation kinetics and feeding strategies in aqueous media. *Biochem. Eng. J.*, 46: 44-53.
- Wang, Z.X. and Y. Shi, 1998. A pH study on the chiral ketone catalyzed asymmetric epoxidation of hydroxyalkenes. *J. Organic Chem.*, 63: 3099-3104.
- Williams, R.C., C.M. Riley, K.W. Sigvardson, J. Fortunak and P. Ma *et al.*, 1998. Pharmaceutical development and specification of stereoisomers. *J. Pharmaceutical Biomed. Analysis*, 17: 917-924.
- Xiao, M.T., Y.Y. Huang, X.A. Shi and Y.H. Guo, 2005. Bioreduction of phenylglyoxylic acid to R(-)-mandelic acid by *Saccharomyces cerevisiae* FD11b. *Enzyme Microbial. Technol.*, 37: 589-596.
- Zilbeyaz, K. and E.B. Kurbanoglu, 2008. Production of (R)-1-(4-Bromo-phenyl)-ethanol by locally isolated *Aspergillus niger* using ram horn peptone. *Bioresour. Technol.*, 99: 1549-1552.
- Zilbeyaz, K., M. Taskin, E.B. Kurbanoglu, N.I. Kurbanoglu and H. Kilic, 2010. Production of (R)-1-phenylethanols through bioreduction of acetophenones by a new fungus isolate *Trichothecium roseum*. *Chirality*, 22: 543-547.