*ORIGINAL ARTICLE*

A C G publications

*Org. Commun.*1:2 (2008) 9-16

organic communications

# Baker's yeast catalyzed asymmetric reduction of methyl acetoacetate in glycerol containing systems

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*(Received June 29, 2008; Revised August 9, 2008; Accepted August 9, 2008)*

**Abstract**: The asymmetric hydrogenation of methyl acetoacetate was successfully performed with baker's yeast in pure glycerol and mixtures of glycerol and water. Though yeast viability was very low after exposure to glycerol, the enzymatic activity in pure glycerol was preserved for some days. In addition, a mixture of glycerol and water combined the advantageous of each individual solvent and resulted in high catalytic performance and efficient product extraction yield.

**Keywords**: Asymmetric synthesis; baker's yeast; glycerol; fermentation; reduction

## 1. Introduction

Chiral hydroxy esters and alcohols are useful intermediates and auxiliaries in the production of various fine chemicals.[1] Since the separation of alcohol racemate is not obvious, catalytic enantioselective hydrogenation of the corresponding prochiral ketones is an attractive route for pure alcohol enantiomers synthesis. Different catalytic systems were reported for this purpose employing both chemo- and bio-catalysts. Noyori's chiral Ru-BINAP complex[2-4] and supported nickel catalyst modified with tartaric acid and sodium bromide[5,6] are some successful systems to note. Yet, employing bio-catalysis for the asymmetric reduction is advantageous since it proceeded at low temperature and in the absence of high hydrogen pressure.[7] Moreover, using the whole cell such as baker's yeast (*Saccharomyces cerevisiae*) for chiral reduction is more attractive from economical, environmental and handling points of view.[8]

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The article was published by Academy of Chemistry of Globe Publications
www.acgpubs.org/OC/index.htm © Published 09/25/2008 EISSN:1307-6175

The asymmetric reduction of β-ketoesters to their corresponding chiral β-hydroxy esters (Fig. 1) was extensively studied with both free and immobilized Baker's yeast cells (FBY and IBY correspondingly).[9,10] Though water is the first solvent of choice for biocatalysis, the low solubility of many organic molecules in water, the existence of undesired side reactions such as hydrolysis, and difficult product separation procedure, limit its applications. Replacing water with organic solvent may overcome these problems and also assist with product recovery. Different organic solvents such as hexane, toluene, ethyl acetate, [9,10] petroleum ether,[11] and liquefied petroleum gas[12] as well as ionic liquids,[13] and fluorous media,[14] which offers recyclable and more environmentally friendly organic media, were tested for this purpose. However, not only that using organic solvent has negative environmental impact, it also affects the yeast cell viability.[9] In addition, glucose which is usually added as carbon and hydrogen source and as electron donor in the regeneration of the co-factor in baker's yeast reductions has negligible solubility in organic solvents.

We recently reported that baker's yeast catalyzed asymmetric reductions of prochiral ketones in glycerol.[15] Glycerol, which is a polar, non-toxic, biodegradable and recyclable liquid manufactured from renewable sources, was used as alternative green organic solvent for several organic reactions.[16] The high polarity of glycerol allowed dissolving glucose or sucrose as energy source. Moreover it allowed easy separation of the product by extraction with glycerol immiscible solvents. It was found that employing both FBY and IBY resulted in high enantioselectivity (>95%) and reasonable activity for various β-keto esters and ketones.

In this paper we report on the asymmetric reduction of methyl acetoacetate (MAA) as representative β-keto ester (Fig. 1) in glycerol and in mixtures of glycerol and water as alternative environmentally friendly solvents. Performing the reactions in these mixtures combined the advantages of each pure solvent. The effect of the glycerol content in the mixture on catalytic performance, yeast viability, and product extraction yield was tested.

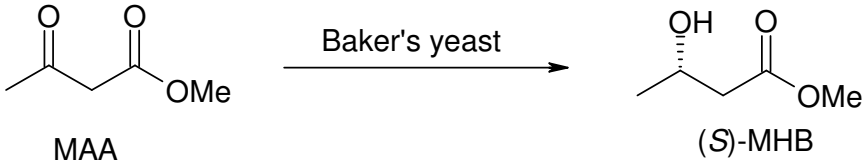


**Figure 1.** Asymmetric reduction of MAA with baker's yeast.

## 2. Results and Discussion

The investigation initiated by proceeding the enantioselective reduction of methyl acetoacetate (MAA) to *(S)*-methyl-3-hydroxybutyrate (*(S)*-MHB, Fig.1) with both FBY and IBY in water and glycerol under similar conditions (Table 1). The enantioselectivity of the product, which is the most important value in asymmetric synthesis, was very high in all the reactions. However, MAA conversion in glycerol was lower than the corresponding conversion in water. Nevertheless, performing the reaction with IBY in glycerol yielded high conversion as in water. It can be attributed to the high amount of water, which was left inside the IBY beads during the preparation procedure,[15,18] as a minimum amount of water (hydration shell) is required around the cells for correct functioning of cell processes and enzyme activities. In addition, the osmotic stress imposed on the free cells by the glycerol probably also contributes to loss of viability and activity.[19] Furthermore, as illustrated in Table 1, addition of sucrose to the reaction mixture in glycerol slightly increased the conversion of MAA hydrogenation. It might be attributed to enhanced regeneration of the co-factor in baker's yeast in the presence of glucose that uses as electron donor.[9-11]

**Table 1**. Comparison of the asymmetric reduction of MAA in water and glycerol.[1]

| Entry | Catalyst | Energy source | Water[2] conversion (%) | Water[2] ee[4] (%) | Glycerol[3] conversion (%) | Glycerol[3] ee[4] (%) |
|---|---|---|---|---|---|---|
| 1 | FBY | No | 98 | >99 | 61 | >99 |
| 2 | IBY | No | 100 | >99 | 87 | >99 |
| 3 | FBY | Sucrose | 100 | >99 | 75 (27[2]) | >99 |
| 4 | IBY | Sucrose | 100 | >99 | 99 (95[2]) | >99 |

[1]50 mL glycerol, 10 g FBY, 1 g MAA, 5 g sucrose, 37°C. [2] 48 h. [3] 96 h.[4] enantiomeric excess=(([*S*]-[*R*])/([*S*]-[*R*]))

The effect of glycerol on FBY viability was thus studied. Cell viability was determined by plate counts of several samples that were taken from the fermentation mixture at different times. As illustrated in Fig. 2, the FBY cell viability was tremendously affected by their exposure to glycerol and dramatic decrees in viability (about 90%) was observed after two minutes. This instant effect is difficult to explain, but it seems that the cells suffered from a shock. The negligible viability in glycerol after short time can be explained by the high osmotic pressure that was imposed on the cell by glycerol.[19] It might result in diffusion of water out of the cells, as detected by Karl-Fischer analysis that might dry the cells and hence affected their viability. The viability of IBY cells, as determined by quality observation, was much higher and similar to the viability in water, even after long time in glycerol. As previously mentioned, the immobilization procedure keeps high amount of water in the beads, which can maintain their viability and thus also their performance.

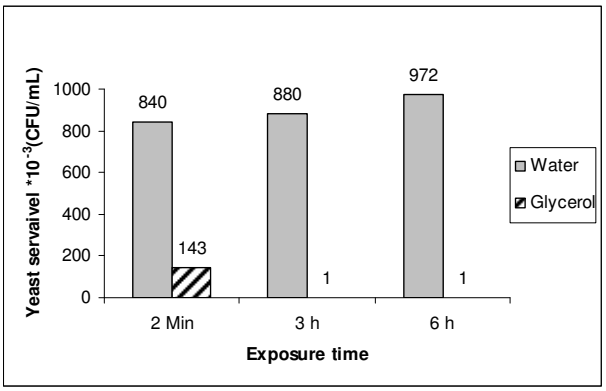


**Figure 2**. Comparison of free baker's yeast viability in glycerol and water.

The poor viability of the yeast in glycerol also raises a question about their ability to carry out biochemical activity. One of the major biochemical activities in yeast is the fermentation of glucose to $CO_2$ and ethanol by the glycolysis pathway. Hence, $CO_2$ production measurement was tested to learn more about the ability of yeast to ferment glucose via glycolysis in glycerol. A close system of two Erlenmeyers, one containing yeast and glucose in glycerol and the other congaing $Ca(OH)_2$ dissolved in water, was used. The Erlenmeyers were connected by a glass pipe, which was dipped in the water solution, and allowed transferring gas from the yeast fermenting side into the second Erlenmeyer. The fermentation was observed by both the formation of foam in the yeast containing Erlenmeyer and the formation of white precipitate, $CaCO_3$, in the second Erlenmeyer as a result of the reaction between calcium hydroxide and $CO_2$.

Although FBY viability in glycerol was very low, simple test of the progress of the reduction of MAA with time showed that the conversion linearly increased with time after one day (Fig. 3). It

illustrates that though the yeast cells were defected after some hours in glycerol the enzymatic activity was preserved. The low conversion during the first day can be explained by the time that required to the enzymes to adapt to the glycerol surrounding.

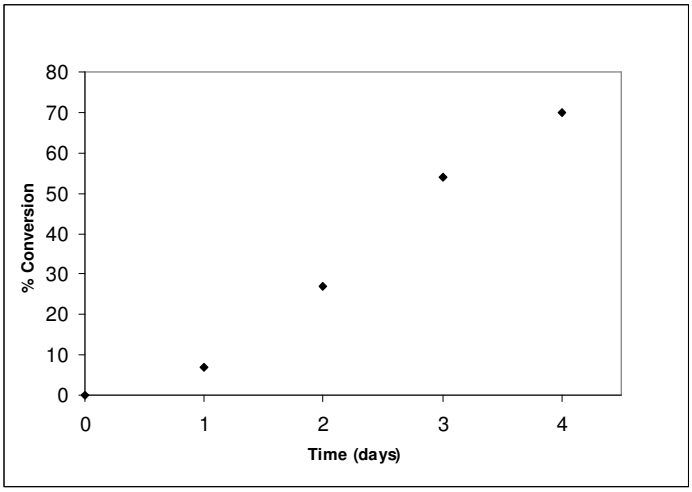


**Figure 3**. Conversion progress in baker's yeast catalyzed asymmetric reduction of MAA. Reaction conditions: 50 mL glycerol, 10 g FBY, 1 g MAA, 5 g sucrose, 37°C.

Finally, employing glycerol as the solvent also allowed easy separation of the product by simple extraction with a glycerol immiscible solvent such as diethyl ether or dichloromethane (Table 2). The effect of the extracting solvent type and extraction procedure on extractions yields of *(S)*-MHB was tested after the reaction reached full conversion. Several representative glycerol and water immiscible solvents were examined as illustrated in Table 2. In general it can be seen that for each solvent the extraction yield with glycerol were slightly higher than the extraction yield with water. In addition, as expected, increasing the extractions steps proportionally increased the product extraction yield (Table 2, entries 3-5). Moreover, in contrast to extraction from water, glycerol did not form emulsion with the extracting solvents and hence the separation was simpler. Although emulsion formation may have also a positive side, such as larger interfacial area for mass transfer and consequently a faster attainment of equilibrium, in this case it did not increased the extraction yield. In addition, extraction of the reaction mixture without pervious separation of the FBY resulted in higher product extractions yields. It is attributed to the fact that part of the organic was left in the yeast cells even at the end of the reaction, as analyzed by extraction of the cell after flirtation from the reaction mixture.

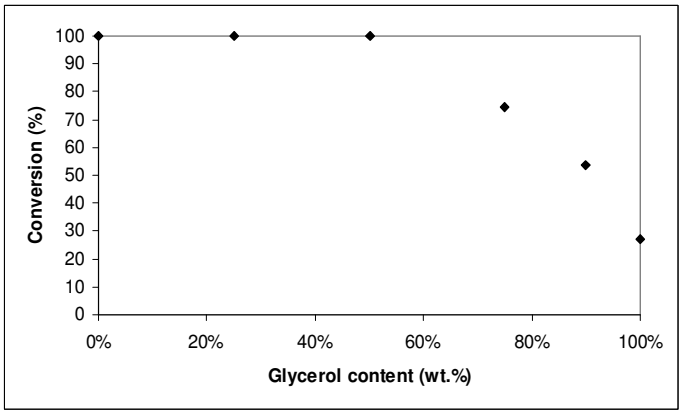


**Figure 4**. MAA conversions in baker's yeast catalyzed asymmetric reduction in water and glycerol mixtures. Reaction conditions: 50 mL solvent, 10 g FBY, 1 g MAA, 5 g sucrose, 37°C, 48 h.

**Table 2.** MHB recovery from glycerol.[1]

| Entry | Extracting solvent | Dielectric constant | Steps | Extraction yield (%) | |
|---|---|---|---|---|---|
| | | | | water | glycerol |
| 1 | n-Hexane | 1.89 | 2 | - | 23 |
| 2 | Diethyl ether | 4.34 | 2 | 32 | 37 |
| 3 | Dichloromethane | 9.08 | 1 | 21 | 24 |
| 4 | Dichloromethane | 9.08 | 2 | 30 | 35 |
| 5 | Dichloromethane | 9.08 | 3 | 65 | 70 |

[1] RT, 40 mL of solvent in each extraction step, 50 mL glycerol, 1 g (*S*)-MHB.

Based on the above results, it is clear that while water is the natural and most suitable solvent for biocatalysis from the viability and activity points of view, glycerol is advantageous in substrate solubility and product separation procedure. Hence performing the asymmetric reduction in a mixture of water and glycerol might combine the advantageous of both individual solvents. To test this hypothesis, the effect of addition of glycerol to water on reaction conversion, yeast viability, and product separation was studied.

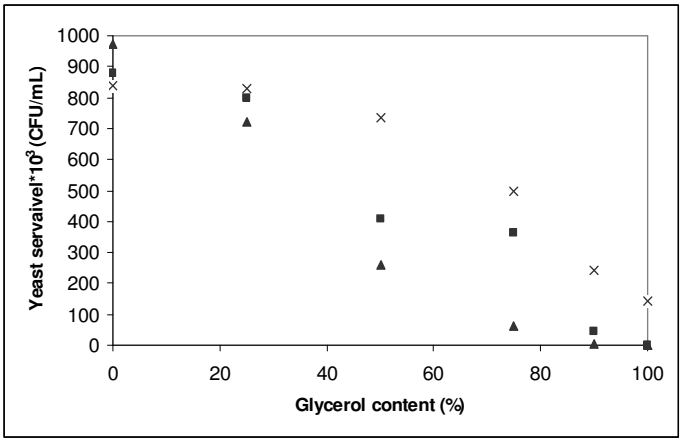


**Figure 5.** Free baker's yeast viability in different glycerol and water mixtures: (x) 2 min; (closed squares) 3 h; (closed triangles) 6h.

First the effect of the amount of glycerol in water on reaction conversion was examined as illustrated in Fig. 4. The results show that increasing the glycerol content up to 50 wt.% in water did not change the conversion. Yet, further increase of the glycerol amount gradually decreased the conversion. The enantioselectivity of the product was not affected by the addition of glycerol and was 99% in all the reactions.

Similar trend was observed in viability measurements (Fig. 5). In general, increasing the amount of glycerol in water behind 30 wt.% decreased the yeast viability. In addition, increasing the exposure time tremendously decreased the viability of the cells behind 30 wt.% of glycerol.

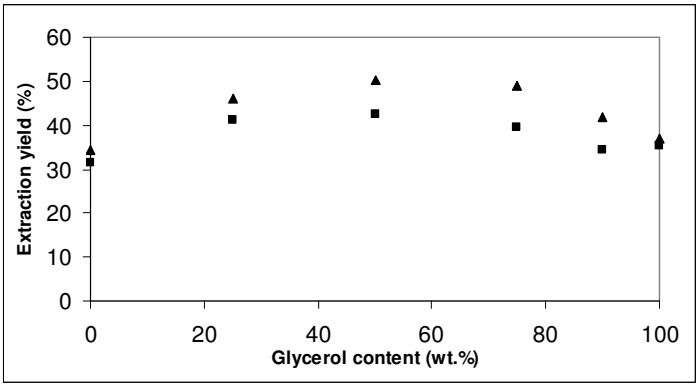


**Figure 6.** MAA extractions yields in water and glycerol mixtures. Extraction conditions: 50 mL solvent, 2 extraction steps with 40 mL extracting solvent, 1 g MAA, RT. (closed triangles) diethyl ether; (closed squares) dichloromethane.

The extractions of a racemic mixture of the product, MHB, that obtained by symmetric reduction with sodium borohydride, were tested with dichloromethane and diethyl ether from several mixtures of glycerol and water (Fig. 6). As illustrated in Fig. 6, employing diethyl ether as extracting solvent resulted in slightly higher extraction yields for all the mixtures. In addition, it was found that higher extractions yields were obtained in mixtures that contained 25-75 wt.% of glycerol in water. In these mixtures, the extractions yields were increased by ~35% when compared to each individual solvent. This maximum can be explained by formation of new interactions between glycerol and water, which decreased the solubility of MHB in the mixture. Similar effect was observed in the extraction of 2-butanol from a mixture of glycerol and water when compare with each pure solvent. While the extraction yield of 1 g of 2-butanol from 50 mL of water or glycerol by 2 steps of 40 mL diethyl ether was 20% and 26% respectively, extracting of 2-butanol from a 50 mL mixture of 50 wt.% glycerol in water resulted in 50% yield, which is 100% higher than the extraction of every individual solvent. It should be mentioned that though 2-butanol has relatively low boiling point it is difficult to distillated it from water since it forms an azeotropic mixture with water. On the other hand the distillation of 2-butanol from glycerol is very easy and efficient.[15]

### 3. Conclusion

To conclude, glycerol, which is a renewable and biodegradable green solvent, was employed as reaction medium in the asymmetric reduction of methyl acetoacetate, a representative β-keto ester, with baker's yeast. Though enantioselectivity was very high and comparable to the reaction in water, the activity was lower. The cell viability in glycerol was very low yet the enzymatic activity was preserved for some days. Performing the reaction in a mixture of glycerol and water combined the advantages of the two solvents and allowed easier and more efficient product extraction.

## 4. Experimental

All chemicals were purchased from Sigma-Aldrich except glycerol (99.5%) that was purchased from Frutarom Ltd, Israel.

### *4.1 Immobilization of baker's yeast*

Immobilized baker's yeast (IBY) were prepared as described by Buque at el. [17]. First, 10 g of FBY (SIGMA, type II) were dispersed in 40 mL of 0.05 M Tris.HCl (pH=8). The dispersion was added to 100 mL of 5 wt.% sodium alginate and mixed for 10 min. Then the resulting mixture was

added dropwise to a 400 mL solution of 0.5 M calcium chloride at 10 °C to produce alginate beads. The beads were left in the solution for 24 h at 4 °C and washed with cold distillate water before use.

### *4.2 Asymmetric reduction*

The asymmetric reduction of MAA with FBY and IBY in glycerol was performed as follows: 10 g of FBY (SIGMA, type II) or 50 g of IBY (prepared from 10 g of FBY) were added to a mixture of 50 mL of solvent in a 250 mL Erlenmeyer and shaked for 30 min (300 rpm and 25 °C). Then 5 g of sucrose were added and the Erlenmeyer was shaken for extra 10 min before 1 g of MAA was added. The reaction mixture was shaken at 300 rpm and 37 °C for 24-96 h. At the end of the reaction the product was extracted with diethyl ether in three steps (3X40 mL). The conversion and the enantiomeric excess of the product were determined by GC analysis with Astec, Chiraldex G-TA® (30 m × 0.25 mm, 0.25 μm thickness).

### *4.3 Viability measurement*

FBY cell viability was determined by plate counting after their exposure to glycerol, mixtures of glycerol and water, and water as reference for different times. The cells were plated onto agar plates after dilution in water and incubated for 96 h at 30°C.

IBY cell viability was tested after their exposure to glycerol by quality observation of growing of cells colonies on agar plates after dissolution of the beads with trisodium citrate and plating it onto agar plates following by incubation for 96 h at 30°C.

### *4.4 Extraction procedure*

Extraction experiments were performed by mixing 50 mL of the solvent, which contained 1 g of methyl 3-hydroxybutyrate (MHB) with 40-120 mL of the extracting solvent in several extraction step, each step contained 40 mL of extracting solvent. The extracting solvent was then evaporated under reduced pressure and the resulting product was analyzed by GC.

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