Kinetic Study of Erythrose Reductase Extracted from *Yarrowia lipolytica*

Masoud Mohammadi Farsani[1], Mohammad Mohammadi[1*], Gholam Reza Ghezelbash[1], Ali Shahriari[2]

[1] Department of Biology, Faculty of Science, Shahid Chamran University of Ahvaz, Ahvaz, Iran
[2]Department of Basic Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

Abstract

Erythritol as a non-caloric and non-cariogenic sweetener is safe for diabetics. Both microbial fermentation and chemical methods can be used to produce erythritol, but chemical methods failed to be industrialized due to their low efficiency. *Moniliella tomentosa, Aureobasidium* sp. and *Yarrowia lipolytica* are industrial producers of erythritol. Erythrose reductase (ER) is a key enzyme in the biosynthesis of erythritol and catalyzes the final step in this pathway. Enzyme extract was obtained from *Y. lipolytica* by grinding cells with 0.5mm glass beads and ER activity was performed using 10 µl enzyme extract, 7.5 mM NADPH and 12 mM D-erythrose in potassium phosphate buffer (pH 7.5). Reaction was monitored with decreasing of NADPH absorbance in OD_{340} at 37 ˚C for 8 min by a microplate analyzer. In order to determine the activation energy (E_a), activity of enzyme was measured in 4-45 ˚C and results were analyzed with Kinetic software according to Arrhenius equation. The best enzyme activity of ER was 6.268 mU. One unit of ER activity was defined as the amount of enzyme that catalyzes the oxidation of 1µmol of NADPH per minute. Specific activity of enzyme was equal to 3.24U/mg and finally the E_a was determined to be 29.6208 KJ.ER specific activity in this study was lower than the only similar study that used *Y. lipolytica*. Purification, overexpression and optimizing the reaction can help to increase enzyme performance.

Keywords: Erythrose reductase, *Yarrowia lipolytica*, Enzyme kinetics

Introduction

Polyols are compatible solutes and effective in osmotic adjustment. Among polyols, glycerol and erythritol are more effective in osmotic adjustment because of their lower molecular weights (Ghezelbash et al., 2012).
Erythritol (MW 122.12) as a non-caloric four-carbon sugar alcohol or polyol is safe for diabetics, cause no gastric side effects and its use in foods and drugs is approved (Ghezelbash et al., 2014; Moon et al., 2010; Tomaszewska et al., 2012) erythritol has 60-70 percent of the sucrose sweetness and both are similar in taste profile (Tomaszewska et al., 2014b) without changing blood glucose and insulin levels, erythritol is excreted in the human urine or by renal processes. Because the bacteria causing dental caries cannot utilize erythritol as a carbon source, it might be safe for human teeth health(Park et al., 2011). Microbial fermentation and chemical methods can be used to produce erythritol. A high-temperature chemical reaction is necessary to synthesis erythritol from dialdehyde starch and this reaction needs a metal as it's catalyzer and therefor the costs of chemical reactions are high(Lee et al., 2010).Nowadays, fermentative methods reach to industrialization due to their high efficiency (Ghezelbash et al., 2014). Producers of erythritol can be osmophilic yeasts belonging to genus *Aureobasidium*, *Candida*, *Moniliella*, *Pichia*, *Pseudozyma*, *Trigonopsis*, *Trichosporon*, *Trichosporonoides* and *Yarrowia* (Moon et al., 2010). A mutant of *Aureobzidium* sp. is being used industrially to produce erythritol at a high yield of 44% in a medium with 40% glucose(Ishizuka et al., 1989). Erythritol can also be produced by the yeast *Yarrowialipolytica* (Rymowicz et al., 2009) which is known as a safe producer of polyols, proteins, lipids and organic acids (Janek et al., 2017). In yeasts, the final step of the pentose phosphate pathway (PPP) is catalyzed by erythrose reductase (ER), a key enzyme to produce erythritol by reducing erythrose. In this reaction, NAD(P)H is used by ER as co-enzyme(Lee et al., 2003b). Finally, yeast uses erythritol as an osmo protectant (Janek et al., 2017). The aim of this study was to determine kinetic parameters of ER and optimizing the enzyme activity.

*Corresponding author E-mail:
mohamadi74@yahoo.com*

Materials and Methods

Microorganism and culture media

Yarrowia lipolytica DSM70562 (Leibniz Institute DSMZ, Germany) was used in this study as an industrial producer of erythritol. The growth medium for activation contained 10% glucose, 0.5% yeast extract, 0.5% KH2PO4, 0.2% (NH4)2SO4 and 0.04% MgSO4(Merck, Darmstadt, Germany). We kept yeast cultures at 4°C and sub-cultured them every 4 weeks. The production medium used for this study contained 1% yeast extract, 0.5% KH2PO4, 0.025% MgSO4, and 20% glucose (Merck, Darmstadt, Germany). The initial pH of both mediums was adjusted to 5.5 (Ghezelbash et al., 2012; Ghezelbash et al., 2014).

Culture conditions

We inoculated a single colony of *Y. lipolytica* into 10 ml of production medium in 100ml Erlenmeyer flask and it was incubated at 30 ˚C and 120 rpm for 48 h. In the next step, we aseptically transferred 2.5 ml of the seed culture into 250ml Erlenmeyer flask containing 50ml of production medium and incubated it at 30 ˚C and 120 rpm for 168 h (Ghezelbash et al., 2012; Ghezelbash et al., 2014).

Preparation of cell extract

Yeast cells from the culture were harvested by centrifugation at 6,000 rpm for 10 min. After washing twice with 50mM potassium phosphate buffer (pH 7.5) containing 5 mM mercaptoethanol, cells were re-suspended in homogenization buffer containing 50 mM potassium phosphate buffer(pH 7.5), 10mMMgCl2, 1mMdithiothreitol (DTT) (Merck, Darmstadt, Germany), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma, Northbrook, USA).In the next step, cell suspension was incubated at room temperature for 1h and then disrupted by grinding with glass beads (0.5 mm in diameter; Sigma, Northbrook, USA) and immediately kept on ice. The ruptured cells were removed by centrifugation at 12,000 rpm for 30 min at 4˚C. The supernatant was used for kinetic assays and further analysis.

Enzyme activity

Erythrose reductase enzyme activity was determined as reported previously (Ghezelbash et al., 2014),with some modifications given below. We determined ER activity using 12mM D-erythrose and 0.75mM NADPH (Sigma Aldrich, Germany) in 50 mM phosphate buffer (pH=7.5) and 10 µl enzyme extract in a microplate chamber (total volume 200 µl). Before reaction, the mixture was kept for 1 min at room temperature to eliminate the endogenous oxidation of NADPH. Then, reaction was monitored for NADPH absorbance in OD_{340} at 37˚C for 15 min using Microplate Analyzer (Bio-Rad, America). One unit of ER activity is the amount of enzyme that catalyzes the oxidation of 1 µmol of NADPH per min at 37 ˚C (Ghezelbash et al., 2014). Enzyme activities were estimated with micro plate analyzer (MPA) software.

Optimization of enzyme activity

Before starting the enzyme kinetic assays, we decided to optimize the reaction by finding the best concentration of NADPH and D-erythrose for determination of optimum enzymatic activity. For this purpose, erythrose reductase activity was investigated at different concentrations of NADPH (0.5 to 2 mM) and D-erythrose (1 to 15 mM).Then the lowest volume of enzyme extract giving highest activity was determined. As the 4th and 5th steps, we estimated the effect of different temperatures (4-40˚C) and the effect of pH (2-9) on enzyme activity respectively. After any step, we used the optimized variable of the previous step.

Enzyme kinetic assay

The kinetic assay was performed using D-erythrose as substrate with concentrations between 0 to 15mM in optimized reaction. Activities of each concentration were measured with MPA software and the Michaelis-Mentenplot, Lineweaver-Burk plot, K_m and V_{max} was obtained with Graph-Pad Prism7 software (Fig.3).

Results

Optimizing the reaction conditions

The best concentration of NADPH with a higher amount in enzyme activity was 1.5mM (Fig.1). Then it was observed that the amounts beyond 10 µl of enzyme extract could not increase the activity of ER. There was no need to determine the optimized concentration of D-erythrose as the substrate of the reaction, because it is used in different concentrations in order to make the Michaelis-Menten plot. (In this study no NADPH to $NADP^+$interconversion activity or vice versa was observed in the crude extract of ER).

Effect of temperature and pH

Optimum assay condition was used to determine the effect of temperature and pH. As showed in Fig. 2, the best activities of ER were in

98

pH=3 and 28˚C
The lowest dose that was able to induce the significant increase in MnBi frequency in this experiment was 1.5 ng.ml^{-1}. To minimize the probability of cell damage, we used this dose throughout the rest of the experiment.
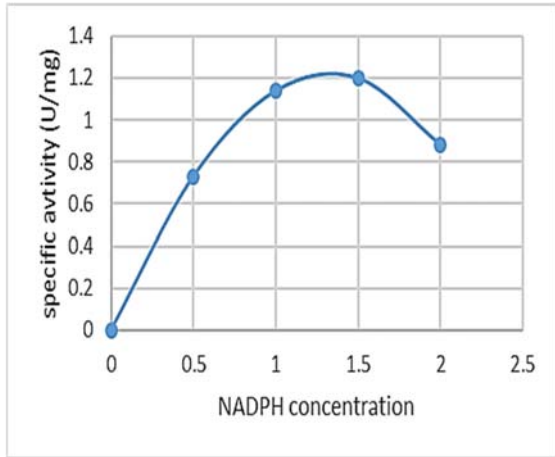

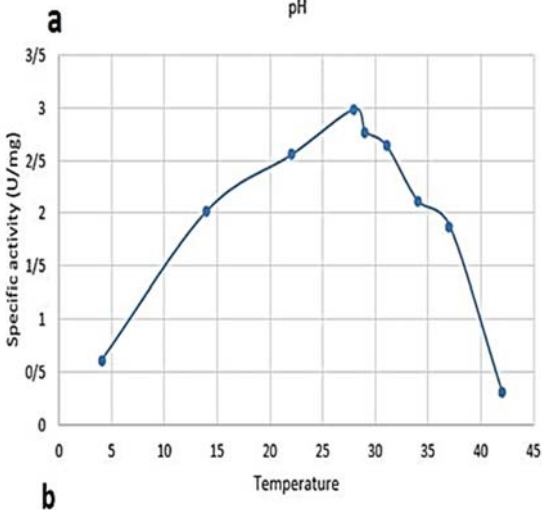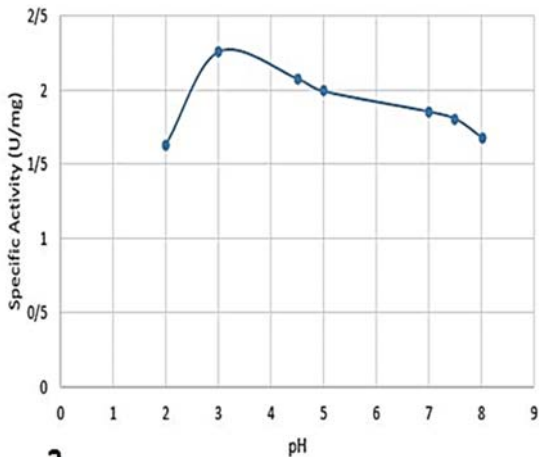**Figure1**. Optimization of NADPH concentraion

Figure 2. Optimization of pH (a) and temperature (b)

Enzyme kinetic parameters

Enzyme kinetic assay was performed using concentrations between 0 to 15mM of D-eryhtrose in optimum conditions of the reaction. An activity was calculated with MPA software and results were analyzed with Kinetic software. Michaelis-Menten plot and Lineweaver-Burk plot are given in Fig.3. Kinetic parameters were determined using nonlinear regression (Table 1).

Table 1. Results obtained from enzyme optimization

Variable	Range	Best activity
NADPH concentration(mM)	0.5 to 4	1.5
D-erythrose concentration(mM)	1 to 15	12
Enzyme extract valume(μl)	5 to 100	10
Effect of Temprature (˚C)	4 to 40	28
Effect of pH	2-9	3

[a]: Statistical difference with control ($P<0.05$)

Discussion

Temperature and pH optimization: the effect of temperature and pH on ER activity revealed that 28℃ and pH=3 are the best conditions for optimum enzyme activity. The best temperature for *Y. lipolytica*'s growth is 28-32℃(Groenewald et al., 2014),but it seems that the best activity of ER should be around 28℃. However, other studies on ER revealed different results. The optimum reported temperature for ER activity in *Candida magnolia* is 40 or 42℃ in different studies(Lee et al., 2010)(Lee et al., 2003a). Recently in a study which used a wild-type of *Y. lipolytica* (A101), results shows the best activity in37℃, but differences between 28℃ and 37℃ was low (only 0.5 U/mg-protein in specific activity) (Janek et al., 2017). As seen in Fig. 2a, the best activity of ER was in pH=3. Higher and lower amounts of pH can significantly decrease the enzymatic activity. As reported before, the optimum activity in low pH can be caused by the phenomenon of high titer of erythritol in acidic pH (Janek et al., 2017). However, producing erythritol by *Y. lipolytica* at low pH has been shown previously (Dobrowolski et al., 2016; Kamzolova et al., 2015; Morgunov et al., 2013; Rymowicz et al., 2009).In another study it has been revealed that the increasing of pH can help decrease amounts of erythritol (Tomaszewska et al., 2014a) and it can be considered as a consequence

99

of ER inactivation (Janek et al., 2017).
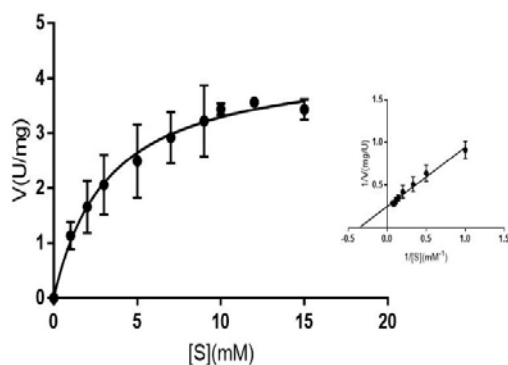
Figure 3. michaelis-menten and Lineweaver-Burk plot

Table 2. Kinetic parameters of erythrose reductase from yarrowia lipolytica

Kinetic parameter	Value (±SEM)	Unit
Km	3.254±0.654	mM
Vmax	4.362±0.2963	U/mg
Kinetic parameter	Value (±SEM)	Unit
The assays were conducted at 28 ℃ and data are presented as the mean ±standard error of the mean, (n=3). Km, Michaelis- Menten constant; Vmax , maximum velocity		

Table 3. Kinetic parameters of erythrose reductase from yarrowia lipolytica

Microorganism	Km (mM)	Reference
*Yarrowia lipolytica*DSM70562	3.254 ± 0.654	-
*Candida magnoliae*JH110	8.5 ± 0.4	(7)
Torula corallina	7.12	(11)
*Aureobasidium*sp. mutant.	8.0	-
Schizophyllum commune	5.0	-
Candida magnoliae	7.9	(13)

Enzyme activity and kinetics :among all substrates which can be used to be oxidized with ER, D-erythrose showed much better activities(Ishizuka et al., 1992; Jovanović et al., 2013; Lee et al., 2010; Lee et al., 2003a; Lee et al., 2003b). In our study, the best initial velocity occurred in 12mM concentration of D-erytrose in the optimum condition. Specific activity of ER

isolated from *Y. lipolytica* in our study was about 60% compare to ER isolated from a wild-type *Y. lipolytica* in a study which used purified enzyme for the reaction (Janek et al., 2017). But in current study we used crude enzyme to promote the reaction and it may be the reason that enzyme activity was lower than the other measurement. In the optimum condition, kinetic parameters (K_M= 3.254 mM and V_{max}=4.362 U/mg protein) were obtained from Prism 7software. Compare to the other studies using different microorganisms containing ER, affinity of the ER isolated from *Y. lipolytica* seems to be more and desirable (Table 3). These properties may be important for using of *Y. lipolytica* in industrial purposes.

Acknowledgments

References

1. Dobrowolski A., Mituła P., Rymowicz W. and Mirończuk A. M. (2016) Efficient conversion of crude glycerol from various industrial wastes into single cell oil by yeast *Yarrowia lipolytica*. Bioresource technology 207:237-243.
2. Ghezelbash G., Nahvi I. and Rabbani M. (2012) Study of polyols production by *Yarrowia lipolytica* in batch culture and optimization of growth condition for maximum production. Jundishapur Journal of Microbiology 5:546-549.
3. Ghezelbash G. R., Nahvi I. and Emamzadeh R. (2014) Improvement of erythrose reductase activity, deletion of by-products and statistical media optimization for enhanced erythritol production from *Yarrowia lipolytica* mutant 49. Current microbiology 69:149-157.
4. Groenewald M., Boekhout T., Neuvéglise C., Gaillardin C., Van Dijck P. W. and Wyss M. (2014) *Yarrowia lipolytica*: safety assessment of an oleaginous yeast with a great industrial potential. Critical reviews in microbiology 40:187-206.
5. Ishizuka H., Tokuoka K., Sasaki T. and Taniguchi H. (1992) Purification and some properties of an erythrose reductase from an *Aureobasidium* sp. mutant. Bioscience, biotechnology, and biochemistry 56:941-945.

6. Ishizuka H., Wako K., Kasumi T. and Sasaki T. (1989) Breeding of a mutant of *Aureobasidium* sp. with high erythritol production. Journal of Fermentation and Bioengineering 68:310-314.

7. Janek T., Dobrowolski A., Biegalska A. and Mirończuk A. M. (2017) Characterization of erythrose reductase from *Yarrowia lipolytica* and its influence on erythritol synthesis. Microbial Cell Factories 16:118.

8. Jovanović B., Mach R. L. and Mach-Aigner A. R. (2013) Characterization of erythrose reductases from filamentous fungi. AMB Express 3:43.

9. Kamzolova S. V., Vinokurova N. G., Lunina J. N., Zelenkova N. F. and Morgunov I. G. (2015) Production of technical-grade sodium citrate from glycerol-containing biodiesel waste by *Yarrowia lipolytica*. Bioresource technology 193:250-255.

10. Lee D. H., Lee Y. J., Ryu Y. W. and Seo J. H. (2010) Molecular cloning and biochemical characterization of a novel erythrose reductase from *Candida magnoliae* JH110. Microbial cell factories 9:43.

11. Lee J. K., Kim S. Y., Ryu Y. W., Seo J.H. and Kim J.H. (2003a) Purification and characterization of a novel erythrose reductase from *Candida magnoliae*. Applied and environmental microbiology 69:3710-3718.

12. Lee J. K., Hong K. W. and Kim S. Y. (2003b) Purification and properties of a NADPHdependent erythrosereductase from the Newly isolated *Torula corallina*. Biotechnology progress 19:495-500.

13. Moon H.J., Jeya M., Kim I.W. and Lee J.K. (2010) Biotechnological production of erythritol and its applications. Applied microbiology and biotechnology 86:1017-1025.

14. Morgunov I. G., Kamzolova S. V. and Lunina J. N. (2013) The citric acid production from raw glycerol by *Yarrowia lipolytica* yeast and its regulation. Applied microbiology and biotechnology 97:7387-7397.

15. Park E. H., Ryu Y. W. and Kim M. D. (2011) Role of osmotic and salt stress in the expression of erythrose reductase in *Candida magnoliae*. Journal of microbiology and biotechnology 21:1064-1068.

16. Rymowicz W., Rywińska A. and Marcinkiewicz M. (2009) High-yield production of erythritol from raw glycerol in fed-batch cultures of *Yarrowia lipolytica*. Biotechnology Letters 31:377-380.

17. Tomaszewska L., Rakicka M., Rymowicz W. and Rywińska A. (2014a) A comparative study on glycerol metabolism to erythritol and citric acid in *Yarrowia lipolytica* yeast cells. FEMS yeast research 14:966-976.

18. Tomaszewska L., Rymowicz W. and Rywińska A. (2014b) Mineral supplementation increases erythrose reductase activity in erythritol biosynthesis from glycerol by *Yarrowia lipolytica*. Applied biochemistry and biotechnology 172:3069-3078.

19. Tomaszewska L., Rywińska A. and Gładkowski W. (2012) Production of erythritol and mannitol by *Yarrowia lipolytica* yeast in media containing glycerol. Journal of industrial microbiology & biotechnology 39:1333-1343.