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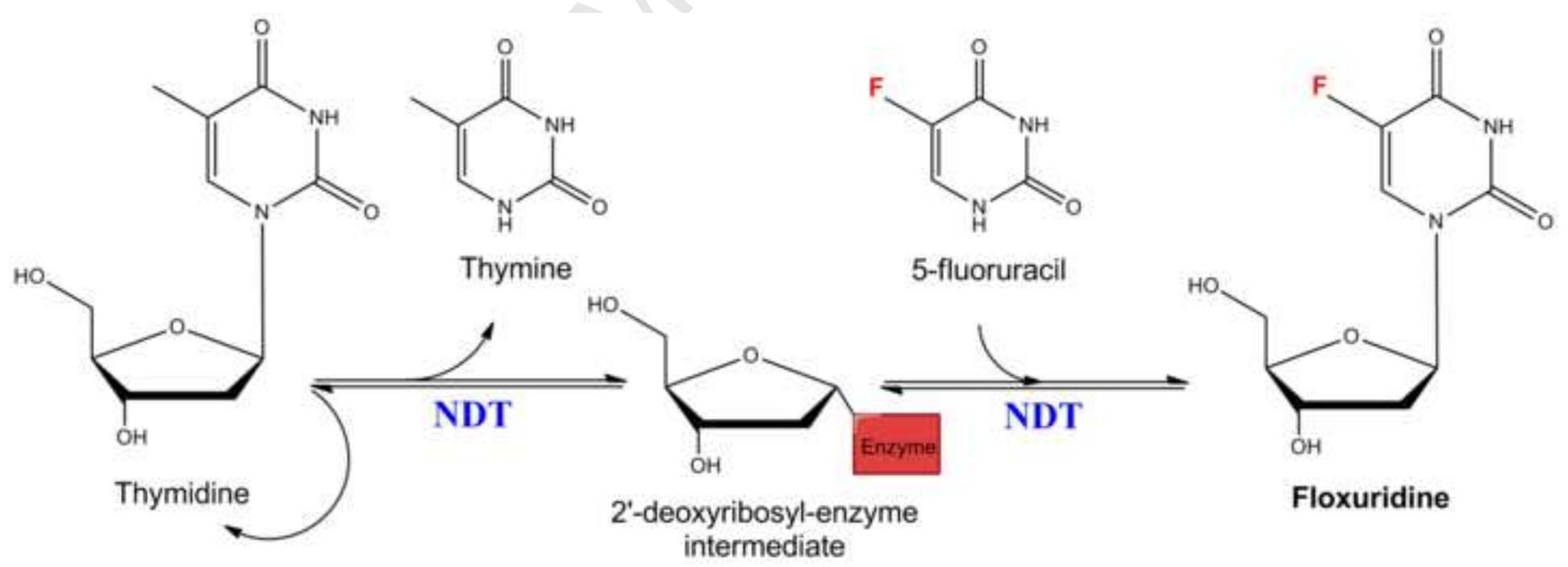
PII: S1381-1177(12)00084-7
DOI: doi:10.1016/j.molcatb.2012.04.004
Reference: MOLCAB 2445

To appear in: *Journal of Molecular Catalysis B: Enzymatic*

Received date: 20-12-2011
Revised date: 29-3-2012
Accepted date: 4-4-2012

Please cite this article as: C.N. Britos, V.A. Cappa, C.W. Rivero, J.E. Sambeth, M.E. Lozano, J.A. Trelles, Biotransformation of halogenated 2'-deoxyribosides by immobilized lactic acid bacteria, *Journal of Molecular Catalysis B: Enzymatic* (2010), doi:10.1016/j.molcatb.2012.04.004

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Highlights

An efficient and green bioprocess is herein reported to obtain halogenated 2'-deoxyribosides by transglycosylation using immobilized lactic acid bacteria.

Lactobacillus animalis ATCC 35046 showed 95 % yield at 0.5 h to synthesize 5-fluorouracil-2'-deoxyriboside

L. animalis ATCC 35046 was able to synthesize 5-halogenated pyrimidine and 6-halogenated purine nucleosides; these results confirm type II NDT activity.

Two laboratory-scale bioprocesses are reported: a continuous system using calcium alginate, and batch bioprocess using DEAE-Sepharose as support.

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Biotransformation of halogenated 2'-deoxyribosides by immobilized lactic acid bacteria

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Keywords

Lactic acid bacteria, 2'-N-deoxyribosyltransferase, whole-cell immobilization, antitumoral compounds.

Abstract

An efficient and green bioprocess is herein reported to obtain halogenated nucleosides by transglycosylation using immobilized lactic acid bacteria (LAB). *Lactobacillus animalis* ATCC 35046 showed a yield of 95 % at 0.5 h to synthesize 5-fluorouracil-2'-deoxyriboside (floxuridine). Calcium alginate was the best matrix for whole-cell immobilization by entrapment. Its productivity was 87 mg/Lh in a continuous bioprocess. When adsorption techniques were evaluated, DEAE-Sepharose was the support which showed higher microbial load, its productivity being 53 mg/Lh. Additionally, this microorganism was able to produce 5-bromouracil-2'-deoxyriboside, 6-chloropurine-2'-deoxyriboside and 6-bromopurine-2'-deoxyriboside.

1. Introduction

Nucleoside analogues such as 5-fluorouracil-2'-deoxyriboside (5FUradRib), better known as floxuridine, have shown activity in patients with colorectal, pancreatic, breast, head and neck cancers [1]. At present, these compounds have been synthesized by chemical methods [2]. Additionally, nucleoside analogue biotransformations can be carried out under very mild conditions, offering environmentally clean technologies [3].

It has been reported that some members of lactic acid bacteria have an enzyme, called 2'-N-deoxyribosyltransferase (NDT, EC 2.4.2.6), which catalyzes the transglycosylation between purine or pyrimidine bases and nucleosides in one-step reaction. NDT has been isolated and characterized in *Lactobacillus helveticus* [4], *Lactobacillus leichmannii* [5], *Lactobacillus reuteri* [6] and *Lactococcus lactis* [7]. Microorganism immobilization is a good way to carry out the bioprocess under preparative conditions. Entrapment and adsorption techniques are the most widely used for whole cell immobilization. The main advantages of this methodology are high operational stability, easy upstream separation and bioprocess scale-up feasibility [8]. Entrapment techniques involve microorganism inclusion in a rigid network to prevent their release into the surrounding medium while allowing mass transfer of substrates and products [9]. In these techniques, the matrix is produced by a physical or chemical polymerization process [10] whereas in adsorption immobilization techniques, microorganisms can be attached to the support by non-specific bonds or affinity interactions [11]. In most cases, these methodologies allow to recover the support after biocatalyst deactivation [12].

In this work, *Lactobacillus animalis* ATCC 35046 was used for 5FUradRib biotransformation. Reaction parameters were optimized in order to obtain an immobilized biocatalyst with improved activity. Additionally, *Lactobacillus animalis* ATCC 35046 was used to obtain other halogenated nucleosides such as 5-bromouracil-2'-deoxyriboside (5BrUradRib), 6-chloropurine-2'-deoxyriboside (6ChPurdRib) and 6-bromopurine-2'-deoxyriboside (6BrPurdRib).

2. Materials & Methods

2.1. Materials

Nucleosides and bases were purchased from Sigma Chem. Co. (Brazil). Culture media compounds were obtained from Britania S.A. (Argentina). Chemicals were purchased from Sigma Chem. Co. (Brazil). Solvents used were of HPLC grade from Sintorgan S.A. (Argentina). Supports (DEAE-Sepharose, EDA-Sepharose, IDA-Agarose, Q-Agarose) were purchased from Sigma Aldrich (Argentina). TLC aluminium sheets (Silica gel 60 F254) were from Merck (Germany).

2.2. Growth conditions

Microorganisms were grown until saturation, harvested by centrifugation during 10 min at 17500 g, washed with 50 mM pH 7 tris(hydroxymethyl)aminomethane-HCl (Tris-HCl) buffer and stored at 4 °C until use. *Lactobacillus* media contained 15 g/L tryptone, 5 g/L soy peptone, 5 g/L NaCl pH 7. *Lactococcus* media: 10 g/L tryptone, 15 g/L meat peptone, 10 g/L yeast extract, 10 g/L glucose, 1 g/L Tween[®] 80 pH 6.5.

2.3. Standard reaction

5FUradRib biotransformation was carried out with 1×10^{10} colony-forming units (CFU), 6 mM thymidine (dThd) and 2 mM 5-fluorouracil (5FUra) in 50 mM pH 7 Tris-HCl buffer at 30 °C and shaking at 200 rpm. Assays were performed three times.

2.4. Lactic acid bacteria selection

Lactococcus (*Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*) and *Lactobacillus* strains (*Lactobacillus acetotolerans* and *Lactobacillus animalis*) were used to assay NDT activity at 3 h in standard conditions [13].

2.5. Sugar donor selection

Different ribosides such as thymidine (dThd), uridine (Urd), 2'-deoxyuridine (dUrd), uracil 1- β -D-arabinofuranoside (araUra) and 2',3'-dideoxyuridine (ddUrd) were assayed as sugar donors. Reactions were made with 1×10^{10} CFU/mL in 1 mL of 2.5 mM riboside and 50 mM pH 7 Tris-HCl buffer, 30 °C at 3 h.

2.6. Optimization of bioprocess parameters

Different reaction parameters such as different growth phases of microorganism (stationary and exponential), buffer concentration (10, 25, 50 and 75 mM) and cation effect (1 and 10 mM Mg^{2+} , K^+ , Zn^{2+} , Ca^{2+} , Cu^{2+} , Mn^{2+} or Ba^{2+}) were studied for 5FUradRib biotransformation in standard conditions.

Additionally, reaction time (0.25, 0.5, 1, 3 and 6 h), amount of microorganisms (1×10^8 , 1×10^9 , 5×10^9 and 1×10^{10}), different reaction temperatures (20, 30, 45 and 60 °C), pH (6, 7 and 8) and different 5FUra and dThd relations (2:2, 6:2 and 2:6 mM) were evaluated.

2.7. Whole cells immobilization

Lactobacillus animalis ATCC 35046 (1×10^{10} CFU) was immobilized by entrapment in agar, agarose, polyacrylamide and sodium alginate as previously described [14]. For adsorption immobilization, 200 mg of EDA-Sepharose, IDA-Agarose, DEAE-Sepharose or Q-Agarose were incubated with 1×10^{10} CFU in 1 mL of 50 mM pH 7 Tris-HCl buffer, during 16 h at 30 °C and shaking at 200 rpm.

2.8. Biotransformation of other halogenated 2'-deoxyribosides

Other purine and pyrimidine bases were tested: 5-bromouracil (5BrUra), 5-chlorouracil (5ChUra), 6-chloropurine (6ChPur), 6-bromopurine (6BrPur) and 6-chloro-2-fluoropurine (6Ch2FPur). The reactions were performed at optimized conditions.

2.9. Analytical Methods

Nucleoside analogue biotransformations were qualitatively evaluated by TLC in 80:20 (v/v) chloroform/methanol as mobile phase. The quantitative analysis was performed by HPLC (Pharmacia LKB) at 254 nm using a Nucleodure 100 C-18 column (5 μ m, 125 mm \times 5 mm). Isocratic mobile phases used were water/methanol at the following ratios: (95:5 v/v) for 5-fluorouracil-2'-deoxyriboside (5FUradRib); (90:10 v/v) for 5-bromouracil-2'-deoxyriboside (5BrUradRib), 6-chloropurine-2'-deoxyriboside (6ChPurdRib), 6-bromopurine-2'-deoxyriboside (6BrPurdRib) and 6-chloro-2-fluoropurine-2'-deoxyriboside (6Ch2FPurdRib) and (99:1 v/v) for 5-chlorouracil-2'-deoxyriboside (5ChUradRib) biotransformations. Product identification was performed by MS-HPLC LCQ-DECAXP4 Thermo Spectrometer with Electron Spray Ionization method (ESI). Phenomenex C18 column (5 μ m, 100 mm \times 2 mm) and Xcalibur 1.3 software (Thermo-Finnigan, USA) were used. Mobile phase used for 5FUradRib (t: 14.0 min, M^+ : 246.9) and 5BrUradRib (t: 34.4 min, M^+ : 308.0) biotransformations was 95/5 (v/v) water/methanol + 0.1% acetic acid and flow was 200 μ L/min. Mobile phase used for 6ChPurdRib (t: 4.2 min, M^+ : 271.8) and 6BrPurdRib (t: 9.4 min, M^+ : 315.8) biotransformations was 15/85 (v/v) water/methanol + 0.1% acetic acid and flow was 200 μ L/min.

3. Results and Discussion

3.1. Lactic acid bacteria selection

Floxuridine biotransformation was assayed to select the best LAB (Scheme 1). Screening was carried out with several *Lactococcus* and *Lactobacillus* strains.

Lactococcus lactis subsp. *cremoris* and *Lactobacillus acetotolerans* showed no significant activity (less than 5 %) whereas *Lactobacillus animalis* ATCC 35046 exhibited the highest NDT activity (48 % at 3 h).

3.2. Sugar donor selection

Urd, dThd, dUrd, ddUrd and araUra were tested as starting ribosides. *Lactobacillus animalis* ATCC 35046 preferentially hydrolyzed dThd and dUrd with yields of 38 % and 35 % at 3 h, respectively. Urd was also hydrolyzed, although with lower yield (22 % at 3 h). However, when ddUrd and araUra were assayed, no hydrolytic activity was detected. In view of these results, dThd was used as starting 2'-deoxyriboside for 5FUradRib biotransformation.

3.3. Optimization of bioprocess parameters

5FUradRib biotransformation from dThd and 5FUra was used to optimize bioprocess parameters at different reaction times.

3.3.1. Effect of microbial growth phase

5FUradRib biotransformation was assayed using *L. animalis* at exponential and stationary microbial growth phases. 5FUradRib conversions were 48 % and 71 % in the exponential and stationary phase at 1 h, respectively. This result could be due to differential expression of NDT, an enzyme involved in the pyrimidine salvage pathway during the stationary growth phase [15].

3.3.2. Effect of Tris-HCl concentration

Different Tris-HCl concentrations were assayed (10 to 75 mM) (Fig. 1A). The highest activity was observed when the buffer concentration was 25 mM (43 mg/Lh), while at lower (10 mM) or higher (50 mM) Tris-HCl concentrations no significant variations in productivity were detected. A decrease in enzyme activity was detected at 75 mM. This buffer effect has already been reported in previous works at values close to 100 mM [16].

3.3.3. Effect of cations

Reactions were carried out with the addition of different cations in the reaction medium and relative activity (ra) was calculated. Results were compared with the control reaction (ra: 1).

When Mn^{2+} and K^+ were added to the reaction medium, 5FUradRib amount decreased 10-fold (ra: 0.10) and 2-fold (ra: 0.50) with respect to control reaction (without cations). The best relative activities (1.22 and 1.30) were achieved when Cu^{2+} and Zn^{2+} were added to the reaction medium (Fig. 1B). However, no significant changes were observed when different alkaline earth cations were tested (Mg^{2+} , Ba^{2+} , Ca^{2+}). In lactic acid bacteria, the ABC transporters activated by Zn^{2+} or Cu^{2+} cations [17] are involved in the internalization of nucleosides and bases [15].

Finally, different cation concentrations (1 and 10 mM) were assayed, showing no significant differences in reaction yields (data not shown).

3.3.4. Effect of the amount of microorganisms

In order to evaluate the optimal quantity of microorganisms for 5FUradRib biotransformation, reactions were performed with increasing amounts of *L. animalis* (Fig. 1C). Biocatalytic activity was detectable with 1×10^9 CFU, reaching a productivity of 13 mg/Lh at 0.5 h of reaction. When the amount of microorganisms was increased 5-fold (5×10^9 CFU), the productivity was 30 mg/Lh at the same reaction time. Moreover, the best productivity (50 mg/Lh) was achieved when 1×10^{10} cells were used. Higher amounts of microorganisms were not evaluated in microscale experiments due to operational difficulties.

3.3.5. Effect of temperature

In the *Lactobacillus* genus, NDT is responsible for transglycosylation when phosphate is absent in the reaction medium and its catalytic activity remains stable up to 50 °C [18]. 5FUradRib biotransformation was performed at different temperatures (20, 30, 45 and 60 °C) using dThd and 5FUra as substrates (Fig. 1D).

5FUradRib conversion values were 94 and 90 % at 30 and 45 °C, respectively.

However, a significant decrease in 5FUradRib yield was observed when the reaction temperature was 60 °C, possibly due to adenine deaminase activity (ADA, EC 3.5.4.2) (Fig. 2) [19]. Therefore, the selected temperature for subsequent reactions was 30 °C.

3.3.6. Effect of pH

5FUradRib biotransformation was carried out at pH 6, 7 and 8. A conversion rate of 51 % at 0.5 h was achieved when the reaction was evaluated under alkaline conditions and this parameter was significantly improved under acid and neutral pH (95 % at 0.5 h). The pKa of 5FUra is 8.0 and alkaline pH values might interfere with an adequate internalization of substrate for subsequent use.

3.3.7. Effect of initial molar ratio (2'-deoxyriboside/ base)

It has been widely reported that transglycosylation reactions are reversible [20]. For this reason, the initial ratio of substrates was analyzed (Fig. 3). When 1:3 and 1:1 dThd/5FUra ratios were assayed, 5FUradRib productivity was 35 and 33 mg/Lh, respectively. However, the amount of 2'-deoxyribosyl-enzyme intermediate available was significantly higher than in previous cases when an excess of dThd was used (3:1 ratio), leading to an increase of 5FUradRib productivity (53 mg/Lh).

Based on these results, the optimal reaction parameters for floxuridine biotransformation were 25 mM Tris-HCl, 1 mM Zn²⁺ (or Cu²⁺), pH 7, 30 °C, 6 mM dThd and 2 mM 5FUra at 0.5 h.

3.4. Microorganism immobilization

3.4.1. Entrapment immobilization

The selected LAB was immobilized by entrapment in thermogels (agar, agarose), ionic gels (alginate) and synthetic polymers (polyacrylamide). These biocatalysts were evaluated for their 5FUradRib biotransformation capacity.

No activity was detected in *L. animalis* immobilized in polyacrylamide, probably due to the toxic effect of acrylamide on microorganisms [21]. Moreover, a release of *L. animalis* into the surrounding medium was observed when this microorganism was immobilized in 2 and 3 % of agar or agarose. However, when 4 % of these gels were used, no microorganism release was observed but conversion values were low.

Finally, immobilizations were made at different alginate concentrations (2, 3 and 4 % w/v). Calcium alginate 4 % w/v was the lowest matrix concentration at which there was no bacterial release. This biocatalyst showed an operational stability of 44 h in batch process. To study the biocatalyst at a pre-pilot scale, 5FUradRib biotransformation was performed in a recycled column with a reaction volume of 12 mL (recirculation velocity: 4.2 mL/min). Under this condition, operational stability was increased more than 3-fold (144 h) and its 5FUradRib conversion was 0.8 mg/L.

3.4.2. Adsorption immobilization

L. animalis immobilization by adsorption was studied on different supports: IDA-Agarose, Q-Agarose, EDA-Sepharose and DEAE-Sepharose.

L. animalis did not significantly attach to IDA-Agarose and Q-Agarose, possibly due to the high negative charge density of IDA-Agarose and the rigid surface structure of quaternary amides in Q-Agarose [22]. However, EDA-Sepharose and DEAE-Sepharose showed satisfactory properties as supports, displaying immobilization yields of 46 and 96 %, respectively. DEAE-Sepharose was selected as support in successive batch experiments.

3.4.3. Comparison of immobilized biocatalysts

5FUradRib biotransformation with *L. animalis* ATCC 35046 immobilized in DEAE-Sepharose and calcium alginate is shown (Table 1). Operational stability of DEAE-Sepharose derivatives was better (248 h) than that of calcium alginate (144 h). However, productivity values were 53 mg/Lh for DEAE-Sepharose and 87 mg/Lh for calcium alginate derivatives. Both immobilized derivatives showed similar yields.

3.5. Biotransformation of other halogenated 2'-deoxyribosides

The possibility of using *L. animalis* to catalyze biotransformations with different purine and pyrimidine bases was evaluated. The products were identified by HPLC-MS. 5BrUradRib biotransformation yield was 22 % in 4 h of reaction using 5BrUra as starting base and dThd as 2'-deoxyribose donor. Additionally, purine bases were used to evaluate the activity and specificity of the biocatalyst. *L. animalis* was able to catalyze 6ChPurdRib (44 %) and 6BrPurdRib (56 %) biotransformation in 4 h of reaction. Activity values were not detectable when using 6Ch2FPur and 5ChUra as starting base (Table 2).

NDTs have been classified into two classes depending on their substrate specificity. Type I NDT is specific for purines and type II NDT is a non-specific enzyme (purines and pyrimidines) [2]. Therefore, the results indicated the presence of NDT II activity in *L. animalis* ATCC 35046.

3.6. Green chemistry parameters

Environment-Factor (E-Factor) is a measure of environmental impact generated by industries. E-factor values are around 25-100 for pharmacological compounds [23]. A low E-Factor value shows mass utilization efficiency and a significant decrease of waste production. In this work, E-Factor values of halogenated nucleoside biotransformations were lower than 7 in all cases.

Carbon efficiency (C-Efficiency) and Atom economy (A-Economy) were designed as parameters to evaluate the efficiency of chemical synthesis. For every biotransformation, C-Efficiency values were greater than 64 % and A-Economy values ranged from 66 to 75 % (Table 3).

4. Conclusions

Lactobacillus animalis ATCC 35046 was selected to produce 5FUradRib. Reaction parameters were optimized to improve productivity and this biocatalyst was stabilized by entrapment and adsorption immobilization techniques. Calcium alginate and DEAE-Sepharose derivatives showed satisfactory 5FUradRib yield and operational stability in batch and continuous bioprocesses.

Additionally, *Lactobacillus animalis* ATCC 35046 was able to synthesize other halogenated nucleosides at short reaction times, and the specificity of purine and pyrimidine bases corresponds to type II NDT activity. These results indicate that *Lactobacillus animalis* ATCC 35046 could be used to produce a broad spectrum of halogenated nucleoside analogues employing an environmentally friendly methodology.

5. Acknowledgments

This research was supported by Ministerio de Ciencia, Tecnología e Innovación Productiva de la Nación Argentina and Universidad Nacional de Quilmes. M.E.L., J.E.S. and J.A.T. are research members of CONICET, C.W.R. is a fellow of CONICET. We are grateful to Carlos Manzione and Carlos Odoguardi (Technical members of CONICET) for their collaboration in some experimental works.

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Scheme 1. Floxuridine biotransformation by *Lactobacillus animalis* ATCC 35046

Figure 1. Optimization of 5FUradRib biotransformation parameters. A: buffer concentration, B: cations (1mM), C: amount of microorganisms, D: temperature

Figure 2. Deaminase activity of *Lactobacillus animalis* ATCC 35046. The activity of adenine deaminase (ADA) was determined by reaction of 2.5 mM adenine in 50 mM Tris-HCl buffer pH 7 at different temperatures

Figure 3. Effect of molar ratio (2'-deoxyriboside/base) on productivity of 5FUradRib biotransformation by *Lactobacillus animalis* ATCC 35046

Table 1. 5FUradRib biotransformation with immobilized *Lactobacillus animalis* ATCC 35046

Support	Yield (mg/L)	Productivity (mg/L h)	Operational stability (h)
Alginate	0.8	87	144
DEAE-Sepharose	0.7	53	248

Table 2. Halogenated 2'-deoxyribosides biotransformation with *Lactobacillus animalis* ATCC 35046

Base	Product	Yield (%)
5BrUra	5BrUradRib	22
5ChUra	5ChUradRib	nd
6BrPur	6BrPurdRib	56
6ChPur	6ChPurdRib	44
6Ch2FPur	6Ch2FPurdRib	nd

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Table 3. Green chemistry parameters of halogenated 2'-deoxyribosides biotransformation with *Lactobacillus animalis* ATCC 35046

Product	E-Factor ^a	C-Efficiency ^b	A-Economy ^c
5FUradRib	3.0	64	66
5BrUradRib	6.1	64	71
6BrPurdRib	1.3	67	75
6ChPurdRib	0.9	67	73

a

$$E - Factor = \frac{\text{total waste mass}}{\text{product mass}}$$

b

$$C - Efficiency = \frac{(\text{carbon atoms in product}) \times 100}{\text{carbon atoms in reactants}}$$

c

$$A - Economy = \frac{(\text{product MW}) \times 100}{\sum(\text{reactants MW})}$$

Figure 1

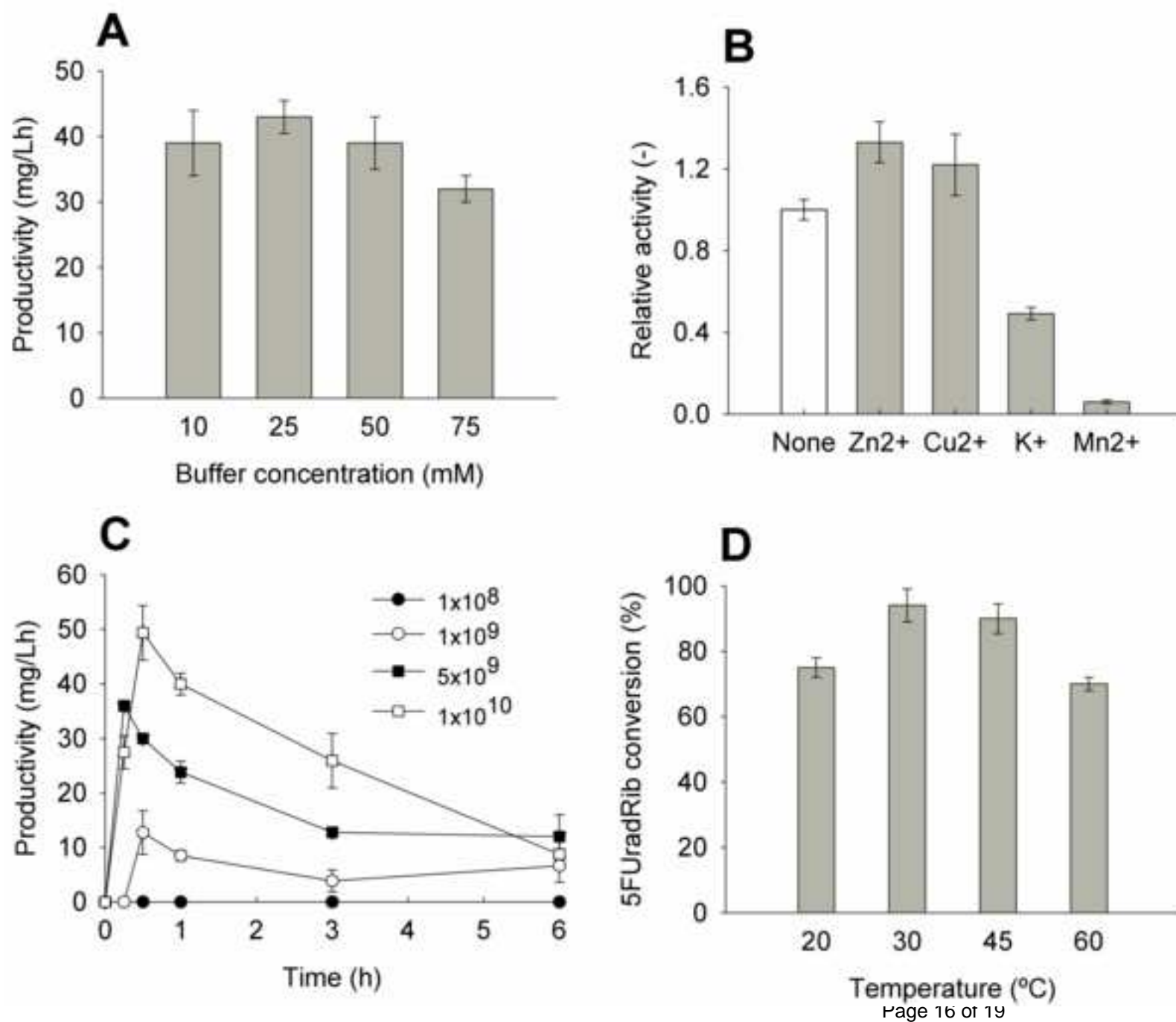


Figure 2

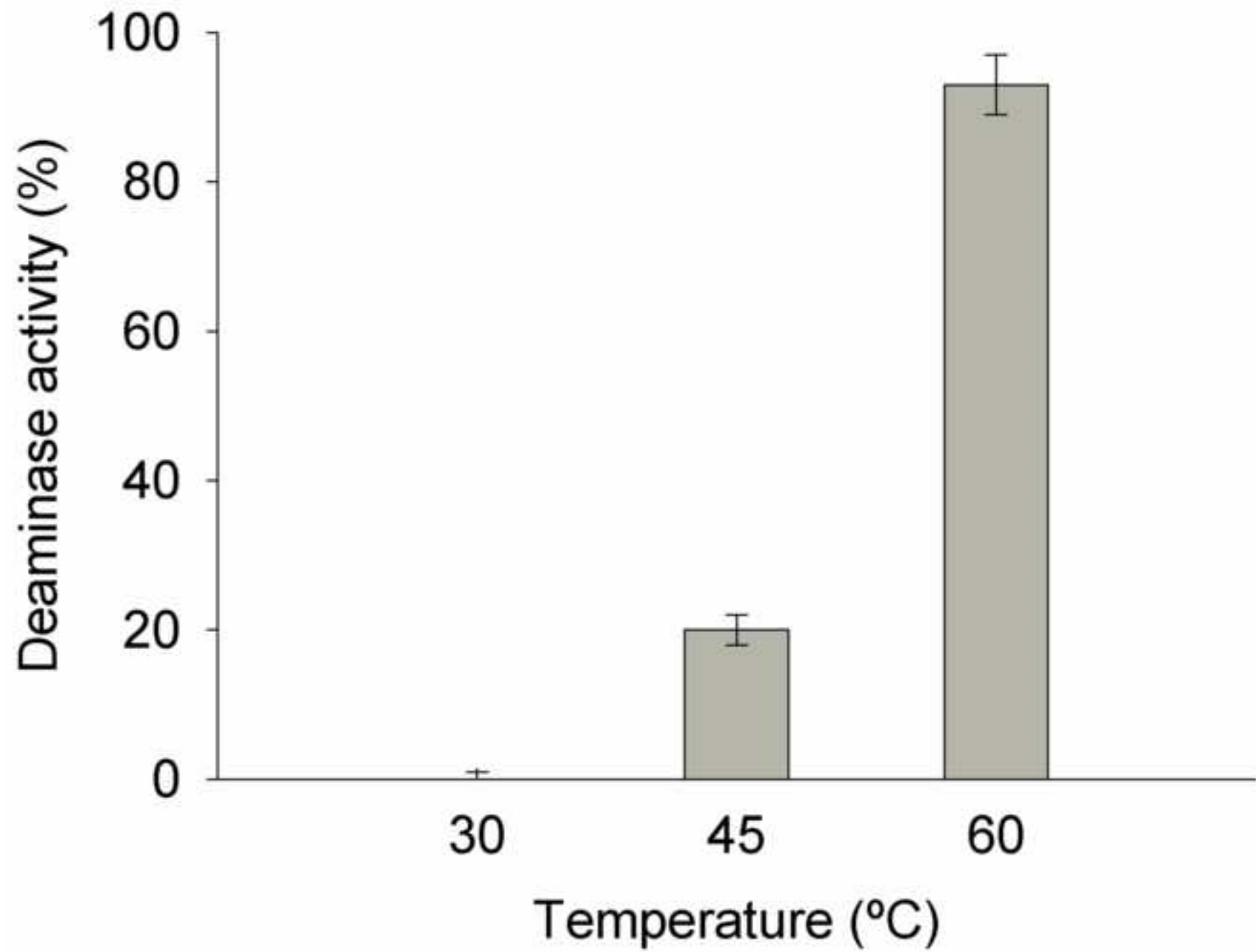


Figure 3

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