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Isolation of *Geobacillus stearothermophilus* L-arabinose Isomerase for the Production of the Novel Food Ingredient D-tagatose

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Abstract

D-tagatose, a low-calorie bulk sweetener, can be produced from D-galactose with an L-arabinose isomerase (L-AI) from *Geobacillus stearothermophilus*. Isolation of L-AI enzyme, intracellularly expressed in *Escherichia coli*, was studied by means of sonication, by chemical cell disruption and by a combined disruption approach. Conditions for cell disruption through sonication, e.g., sonication time, duty cycle, power and sample volume were determined for the wild type (WT) enzyme which was produced without an inducible expression system. The highest release was observed at a power of 120 W after 5 minutes of sonication with a duty cycle of 50% and sample volume of 60.0 mL resulting in a relative L-AI activity of 92.5 ± 0.9%. Chemical cell disruption with 16.5 mM triton X-100 for 17 hours gave even better results compared to sonication, namely, a relative L-AI activity in the crude extract of 95.6 ± 1.2%. Additional experiments were performed on cell disruption of the wild type enzyme produced with an inducible expression system (WT_i). Treatment of WT_i enzyme with 16.5 mM triton X-100 for 17 hours led to a lower relative L-AI activity, namely, 84.0 ± 0.5%. A combined approach of a prior chemical lysis with triton X-100 followed by

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sonication led to a further increase of L-AI activity towards 89.6 ± 0.3%.

Keywords: D-tagatose, L-arabinose isomerase, *Geobacillus stearothermophilus*, Cell disruption, Sonication, Triton X-100

1. Introduction

During recent years, D-tagatose has attracted a lot of attention due to its wide range of healthy benefits. D-tagatose is a naturally occurring bulk sweetener exhibiting a low caloric value of 1.5 kcal g^{-1} and approximately 92% of the sweetness of sucrose (Kim, 2004; Liang et al., 2012; Lu et al., 2008; Oh, 2007; Skytte, 2006; Vastenavond et al., 2012). Furthermore, D-tagatose has a low glycaemic response, prebiotic properties and does not cause tooth decay. These benefits make D-tagatose highly suitable for utilization in healthy food and nutrition for diabetics (Liang et al., 2012; Lu et al., 2008; Oh, 2007; Skytte, 2006; Vastenavond et al., 2012).

D-tagatose can be produced either chemically or biochemically from D-galactose (Cheetham & Wootton, 1993; Jorgensen et al., 2004; Kim et al., 2001; Kim et al., 2003; Kim, 2004; Oh, 2007; Skytte, 2006; Vastenavond et al., 2012). Chemical conversion of D-galactose is effectuated under alkaline conditions, in the presence of calcium hydroxide and a catalyst (Cheetham & Wootton, 1993; Jorgensen et al., 2004; Kim, 2004; Vastenavond et al., 2012). Chemical production of D-tagatose is however not economically feasible due to by-product formation and complex purification steps (Jorgensen et al., 2004; Kim et al., 2003). Due to the negative aspects of chemical D-tagatose production, a biochemical process is preferred. D-galactose can be isomerised to D-tagatose with an L-arabinose isomerase (L-AI) enzyme (EC 5.3.1.4) from *Geobacillus stearothermophilus* (Jorgensen et al., 2004; Kim et al., 2001; Kim, 2004; Vastenavond et al., 2012). The L-AI is expressed in the host organism *E. coli*, which implies an intracellular L-AI expression (Jorgensen et al., 2004; Kim & Oh, 2005; Lee et al., 2004; Lee et al., 2005; Liang et al., 2012; Rhimi & Bejar, 2006).

For application of free or immobilised L-AI within the production process of D-tagatose, the enzyme needs first to be isolated in case of an intracellular expression system. Methods for cell disruption can be divided into mechanical and non-mechanical methods. Mechanical disruption methods such as sonication are non-specific, this in contrast to non-mechanical procedures (Falconer et al., 1997; Geciova et al., 2002; Harrison, 1991; Middelberg, 1995; Seddon et al., 2004). Cell disruption through sonication can be attributed to shear forces, shock waves and heating. Ultrasound can potentially also cause inactivation of enzymes. The major cause of this enzyme inactivation is a free-radical production (Alzamora et al., 2011; Ananta et al., 2005; Bermúdez-Aguirre et al., 2011; Mawson et al. 2011; Santos et al., 2009). The efficiency of sonication depends on several parameters e.g. sonication time, duty cycle, power and volume (Agrawal & Pandit, 2003; Benov & Al-Ibraheem, 2002; Feliu et al., 1998; Geciova et al., 2002; Joyce et al., 2003; Kapucu et al., 2000; Kuboi et al., 1995; Özbek & Ülgen, 2000). For each parameter, a compromise needs to be made between maximal cell disruption and minimal enzyme inactivation. Non-mechanical methods can be divided into physical, enzymatic and chemical techniques. A broad variety of chemical components can be used for chemical cell disruption, e.g., antibiotics, chelating agents, detergents, chaotropes, solvents, hydroxides or hypochlorites (Falconer et al., 1997; Geciova et al., 2002; Harrison, 1991; Middelberg, 1995; Seddon et al., 2004).

In this study, several methods for cell disruption of *E. coli* for isolation of the L-AI from

Geobacillus stearothermophilus were investigated, more specific mechanical disruption by sonication and chemical cell disruption with triton X-100, tween80, urea and isopropanol, respectively. Moreover, the effect of combining chemical lysis and sonication on L-AI activity was investigated. The order of applying different techniques could also play an important role. Chemical treatment can damage the cell wall so that sonication is facilitated. On the other hand, chemical reagents can, next to cell disruption, partially solubilise inclusion bodies which are accessible after cell disruption through sonication (Feliu et al., 1998; Ho et al., 2006).

2. Material and Methods

2.1 Cultivation of E. coli and Expression of L-AI Enzyme

The L-AI enzyme from *Geobacillus stearothermophilus* was expressed intracellularly in *E. coli* BL21. The wild type (WT) strain was supplied by the Center for Protein Engineering (CIP, University of Liège, Belgium) and lacks the need for induction through the removal of the LacI repressor. Cells were cultivated in a bioreactor (New Brunswick Scientific co., inc. U.S.A. BioFlo IV) containing 7.0 L cultivation medium consisting of 16.8 mL L^{-1} glycerol [95% (v/v)] (Merck), 10.0 g L^{-1} yeast extract (Organo Technie), 15.0 g L^{-1} K_2HPO_4 (Caldic), 7.50 g L^{-1} KH_2PO_4 (Caldic), 3.00 g L^{-1} $(NH_4)_2SO_4$ (Caldic), 2.20 g L^{-1} Na-citrate (Caldic), 1.50 g L^{-1} $MgSO_4 \cdot 7H_2O$ (Caldic), 0.197 g L^{-1} $MnCl_2 \cdot 4H_2O$ (Chem-Lab), 0.0125 g L^{-1} chloramphenicol (Sigma-Aldrich) and 2.00 mL L^{-1} trace solution. The trace solution consisted of 33.8 g L^{-1} $Zn(CH_3COO)_2 \cdot 2H_2O$ (VWR), 14.1 g L^{-1} Na-EDTA$\cdot 2H_2O$ (VWR), 3.00 g L^{-1} H_3BO_3 (Alfa Aesar), 2.50 g L^{-1} $CoCl_2 \cdot 6H_2O$ (Alfa Aesar), 2.10 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$ (VWR), 1.50 g L^{-1} $CuCl_2 \cdot 2H_2O$ (VWR) and 0.197 g L^{-1} $MnCl_2 \cdot 4H_2O$ (Chem-Lab). The temperature was set at 25°C and the pH was controlled at 7.0 with 24.5% NH_3 (v/v) (Brenntag). When glycerol was depleted, a concentrated feed was added to the bioreactor at a flow rate of 1.2 mL min^{-1}. The feed consisted of 365 mL L^{-1} glycerol [95% (v/v)] (Merck), 178 g L^{-1} yeast extract (Organo Technie), 1.00 g L^{-1} $MgSO_4 \cdot 7H_2O$ (Caldic), 0.394 g L^{-1} $MnCl_2 \cdot 4H_2O$ (Chem-Lab), 0.0250 g L^{-1} chloramphenicol (Sigma-Aldrich) and 1.00 mL L^{-1} trace solution. After 72 hours of fermentation, an optical density (OD) of 111 and dry matter content of 3.67% (w/v) was reached. The effect of sonication time, duty cycle, power and sample volume on the efficiency of sonication as well as the screening of chemical components was investigated with the WT strain.

The effect of treatment time and concentration of triton X-100 as well as the combined disruption approaches on isolation of L-AI was investigated with *E. coli* BL21 with the wild type enzyme, which was produced with an inducible expression system (WT_i). This strain, also supplied by the CIP, is different from the previous strain because it needs to be induced with isopropyl β-D-1-thiogalactopyranoside (IPTG). Furthermore, the carbon source during the cultivation was 20 g L^{-1} glucose (Tereos Syral) instead of glycerol. The feed contained 354 g L^{-1} glucose (Tereos Syral). The temperature before induction was set at 30°C. After induction, the temperature was lowered to 25°C. After 50 hours of fermentation, an OD of 107 and dry matter content of 3.87% (w/v) was reached.

Cells were harvested by centrifugation (Awel centrifugation) at 4°C for 30 minutes at 4000

rpm and were washed with tris-HCl buffer [pH of 7.0, adjusted with 37% (w/w) HCl (VWR)] consisting of 50.0 mM tris(hydroxymethyl)aminomethane) (tris, Sigma-Aldrich) and 1.00 mM $MnCl_2.4H_2O$ (Chem-Lab). Next, cells were centrifuged (Awel centrifugation) at 4°C for 30 minutes at 4000 rpm. Finally, the washed pellet was diluted to the initial cell volume with tris-HCl buffer of pH 7.0. Afterwards, the D-tagatose production was determined. 0.500 mL intracellular enzyme was added to 1.500 mL D-galactose solution [350 g L^{-1} D-galactose (Inalco), 50.0 mM tris, 1.00 mM $MnCl_2.4H_2O$] of pH 7.0 [adjusted with 37% (w/w) HCl]. Mixtures were incubated for 2 hours at 60°C in a water bath with shaking mechanism (Heto Birkerod Denmark). Subsequently, the D-tagatose concentration was determined with High-Performance Liquid Chromatography (HPLC) with Refractive Index (RI) detection. Determination of the D-tagatose concentration with HPLC was performed on a Bio-Rad Aminex Carbohydrate HPX-87P column (300 × 7.8 mm). Samples were pretreated with 20 µL mL^{-1} Carrez I and II (VWR) in order to remove the proteins. Similar L-AI activities were found for the WT enzyme and WT_i enzyme, namely 30.2 ± 2.0 and 29.0 ± 0.3 g L^{-1} D-tagatose, respectively.

2.2 Cell Disruption by Sonication

Cell disruption was performed at 20 kHz with a Branson Sonifier 250 equipped with a horn tip. The washed pellet of the WT enzyme was used for these experiments. Different parameters were tested including sonication time, duty cycle, power and sample volume. Sonication time was varied from 3 minutes to 10 minutes and the duty cycle was changed from 10 to 100%. A duty cycle of 50% implies 0.5 seconds of pulsation and 0.5 seconds of rest. Power and sample volume were varied from 60 to 120 W and 30.0 to 120 mL, respectively. All sonication experiments were performed on ice and were repeated four times. After cell disruption, cell suspensions were centrifuged (Awel centrifugation) at 4°C for 30 minutes at 4000 rpm and the crude extract was separated from the cell debris. In the crude extract, the protein concentration and the relative L-AI activity was determined. The cell debris was washed with tris-HCl buffer followed by centrifugation for 30 minutes at 4000 rpm. Finally, the cell debris was diluted to the initial cell volume with tris-HCl buffer of pH 7.0 and the corresponding OD was measured. The mean value was calculated for the protein concentration, relative L-AI activity and OD and corresponding standard deviations were calculated.

2.3 Chemical Cell Disruption

For chemical cell disruption, initially different reagents were screened, namely 16.5 mM triton X-100 (Sigma-Aldrich), 8.17 mM tween80 (Merck), 2.00 M urea (Merck) and 1.31 M isopropanol (Merck). 50.0 mL washed pellet of the WT enzyme was incubated with these chemical reagents for 17 hours at 25°C and mixtures were stirred at 200 rpm (IKA-Werke). All experiments were performed four times. After cell disruption, cell suspensions were post-treated in accordance to the sonication experiments. In addition, the effect of triton X-100 concentration was investigated by incubating 50.0 mL washed pellet of WT_i enzyme with 16.5, 33.1 and 49.6 mM triton X-100 at 25°C for 17 hours and mixtures were stirred at 200 rpm. The treatment time in the presence of 16.5 mM triton X-100 was varied between 6,

17 and 28 hours at 25°C and mixtures were stirred at 200 rpm. All experiments were performed three times. After cell disruption, cell suspensions were post-treated in accordance to the sonication experiments.

2.4 Combined Disruption Approach

The effect of sonication followed by chemical treatment was evaluated by incubating washed pellet of WT_i enzyme after sonication with 16.5 mM triton X-100 for 6 hours at 25°C and mixtures were stirred at 200 rpm. Sonication was performed at 120 W during 5 minutes with a duty cycle of 50% and sample volume of 60.0 mL. The effect of a prior chemical lysis and subsequent sonication treatment was also investigated under similar operational conditions. After cell disruption, cell suspensions were post-treated in accordance to the sonication experiments. Results of the combined approaches were compared to the results of the individual disruption techniques.

2.5 Protein Quantification

The protein concentration was determined using the Bicinchoninic Acid Protein Assay Kit (BCA1, Sigma-Aldrich). 2.00 mL bicinchoninic acid (BCA) working reagent was added to 0.100 mL bovine serum albumin (BSA) standard (0, 200, 400, 600, 800 and 1000 µL mL^{-1} BSA), blank or unknown sample in an appropriate dilution. Mixtures were incubated for 2 hours at 25°C. After 2 hours, the absorbance was measured at 562 nm and protein concentrations were calculated according to the standard curve.

2.6 Determination of Relative L-AI Activity

To determine the L-AI activity, 0.500 mL crude extract was added to 1.500 mL D-galactose solution [350 g L^{-1} D-galactose, 50.0 mM tris, 1.00 mM $MnCl_2.4H_2O$] of pH 7.0 [adjusted with 37% (w/w) HCl]. Mixtures were incubated for 2 hours at 60°C in a water bath with shaking mechanism (Heto Birkerod Denmark). Subsequently, the D-tagatose concentration was determined with the cysteine-carbazole method (Dische & Borenfreund, 1951) or with High-Performance Liquid Chromatography (HPLC) with Refractive Index (RI) detection. With the cysteine-carbazole method, the reaction of the ketose, D-tagatose, with cysteine and carbazole in an acidic environment results in the formation of a purple product. The absorbance was measured at a wavelength of 560 nm with a Cary 100 Bio UV-Visible spectrophotometer from Varian. The relative L-AI activity was determined as follows:

$$Relative\ L-AI\ activity\ (\%) = \frac{activity\ of\ crude\ extract\left(\frac{U}{ml}\right)}{activity\ of\ cells\left(\frac{U}{ml}\right)} x100$$

2.7 Determination of Optical Density

The OD was determined by measuring the absorbance at a wavelength of 600 nm with a Cary 100 Bio UV-Visible spectrophotometer from Varian, after appropriate dilution.

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3. Results and Discussion

3.1 Effect of Sonication Time

In this study, several parameters that influence the efficiency of cell disruption were investigated for isolation of *Geobacillus stearothermophilus* L-AI from *E. coli* in the context of the production of D-tagatose from D-galactose. The parameters were examined by sonication of washed pellet of WT type enzyme. The efficiency of sonication was evaluated based on the activity and protein concentration of the crude extract as well as the OD of the cell debris.

At first, the influence of sonication time on isolation of L-AI from *Geobacillus stearothermophilus* expressed in *E. coli* was studied at 120 W with a duty cycle of 50% and 60.0 mL sample volume. The sonication time was varied from 3 to 10 minutes. In order to evaluate the effect of the sonication time, the mean value of the protein concentration and relative L-AI activity in the crude extract and OD of the cell debris as well as the standard deviations were determined. Results are shown in Figure 1.

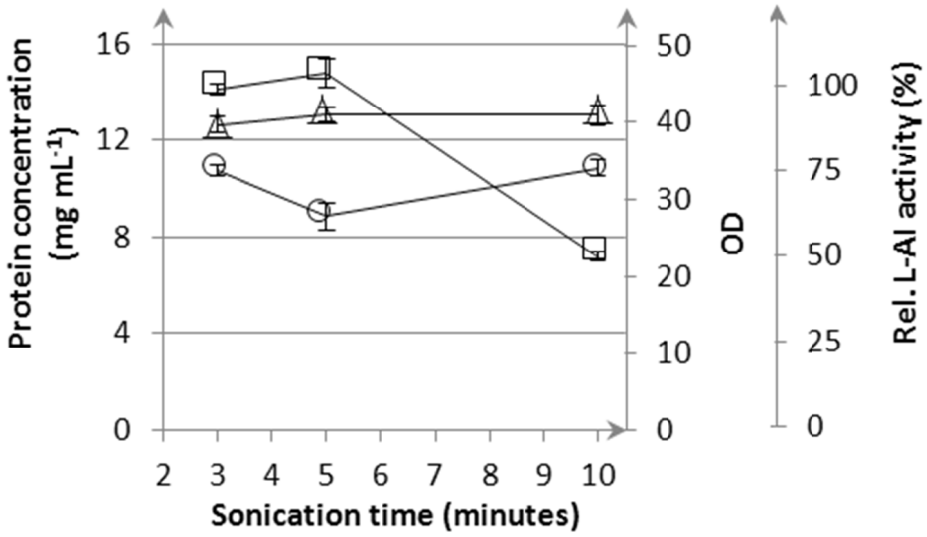

Figure 1. Effect of sonication time on the protein concentration (□) and relative L-AI activity (Δ) of the crude extract and OD of the cell debris (○)

A slightly higher protein concentration and lower OD of the cell debris was found when increasing sonication time from 3 to 5 minutes. The relative L-AI activity in the crude extract after 3 and 5 minutes of sonication was 89.5 ± 1.1% and 92.5 ± 0.9%, respectively. Thus, an increase of the sonication time from 3 to 5 minutes led to an augmentation of the relative L-AI activity in the crude extract. These results confirm the higher protein concentration found in the crude extract after 5 minutes. A further increase of the sonication time up to 10 minutes did however not lead to a significant increase of the relative L-AI activity when

compared to 5 minutes sonication. As it can be seen from the results in Figure 1, a strong decrease in protein concentration was noticed when increasing the sonication time from 5 to 10 minutes. The decline in protein concentration in the crude extract can be explained by inactivation of the released enzyme which resulted in an augmentation of the OD of the cell debris.

For all sonication times, a relative L-AI activity of approximately 50% was still noticed in the cell debris after cell disruption (data not shown). This is either due to an incomplete cell disruption or to the presence of inclusion bodies (Baneyx & Mujacic, 2004; Sabate et al., 2010). The use of *E. coli* as host organism has several advantages, e.g., growth on inexpensive carbon sources and rapid biomass accumulation. However, it is not uncommon that recombinant proteins in *E. coli* fail to from a correct conformation and associate with each other to form insoluble aggregates known as inclusion bodies (Baneyx & Mujacic, 2004; Sabate et al., 2010).

3.2 Effect of Duty Cycle

In the next phase, the effect of the duty cycle on the disruption performance was evaluated. Sonication was performed at 120 W for 5 minutes with 60.0 mL sample at various duty cycles, e.g., 10, 25, 50, 75 and 100%. The effect of the duty cycle was examined by measuring the mean value of the protein concentration and relative L-AI activity in the crude extract and the OD of the cell debris as well as the standard deviations. The results are illustrated in Figure 2.

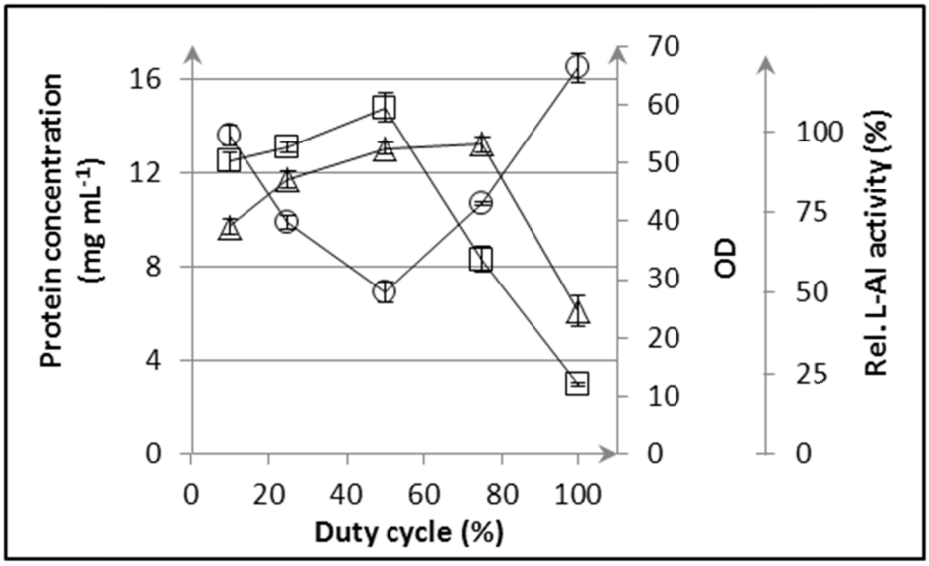

Figure 2. Effect of duty cycle on the protein concentration (□) and relative L-AI activity (Δ) of the crude extract and OD of the cell debris (○)

The increase of the duty cycle from 10 till 50% had a positive effect on the protein

concentration and relative L-AI activity in the crude extract. An increase of the relative L-AI activity in the crude extract was observed, from 68.8 ± 1.4 % to 92.5 ± 0.9%. These results are in accordance to the decrease of OD of the cell debris. A further increase of the duty cycle to 75 and 100% negatively influenced the protein concentration in the crude extract. At the same time, an increase of the OD of the cell debris was observed which can be correlated to the inactivation of released proteins after cell disruption. Accordingly, highest isolation of active L-AI enzyme was achieved with a duty cycle of 50%.

3.3 Effect of Power

The effect of the power on isolation of L-AI enzyme expressed in *E. coli* was investigated in this study within a range of 60 till 120 W. Duty cycle, sonication time and sample volume were set at 50%, 5 minutes and 60.0 mL, respectively. Figure 3 shows the results of the effect of the power on isolation of the L-AI enzyme.

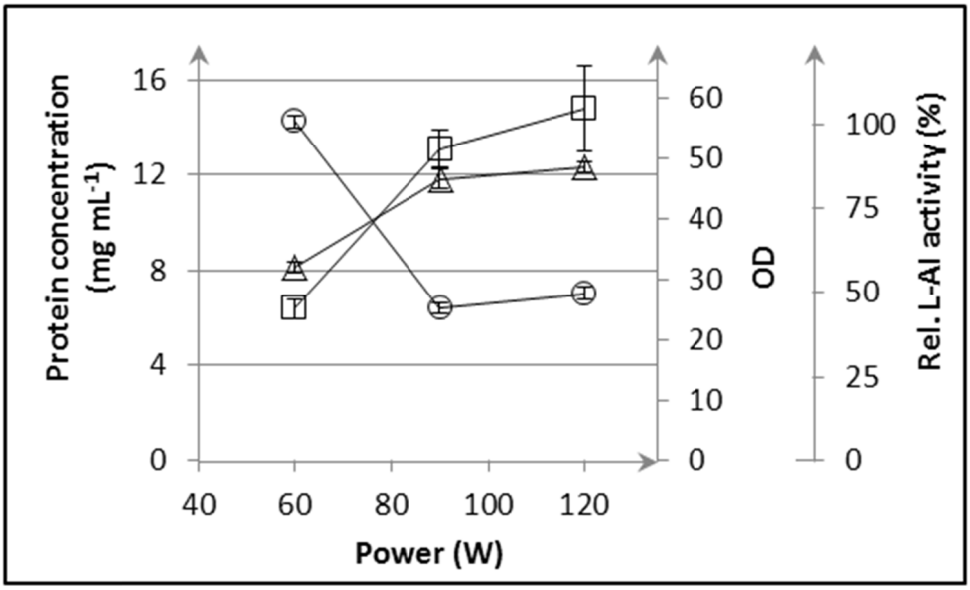

Figure 3. Effect of the power on the protein concentration (□) and relative L-AI activity (Δ) of the crude extract and OD of the cell debris (○)

By altering the power from 60 to 90 W, a strong increase in protein concentration was noticed. These data were confirmed by the decrease of the OD in the cell debris. These results were in accordance with the data of Kapucu et al. (2000), who investigated ultrasonic disruption of the gram negative bacterium *Acetobacter peroxdans*. The results in figure 3 show that a further increase of the power towards 90 and 120 W led to a protein concentration of 13.1 ± 0.6 and 14.8 ± 0.6, respectively. Minor changes in OD were observed with an increase in power from 90 to 120 W. A power increase from 60 to 120 W contributed to an increase of the relative L-AI activity from 61.0 ± 0.9 % to 92.5 ± 0.9%.

3.4 Effect of Sample Volume

The effect of sample volume on the release of L-AI was studied at a constant power of 120 W, a duty cycle of 50% and a sonication time of 5 minutes. The sample volume was varied from 30.0 to 120.0 mL. Measurement of the protein concentration and relative L-AI activity in the crude extract and OD of the cell debris was carried out for studying the effect of sample volume on cell disruption (Figure 4).

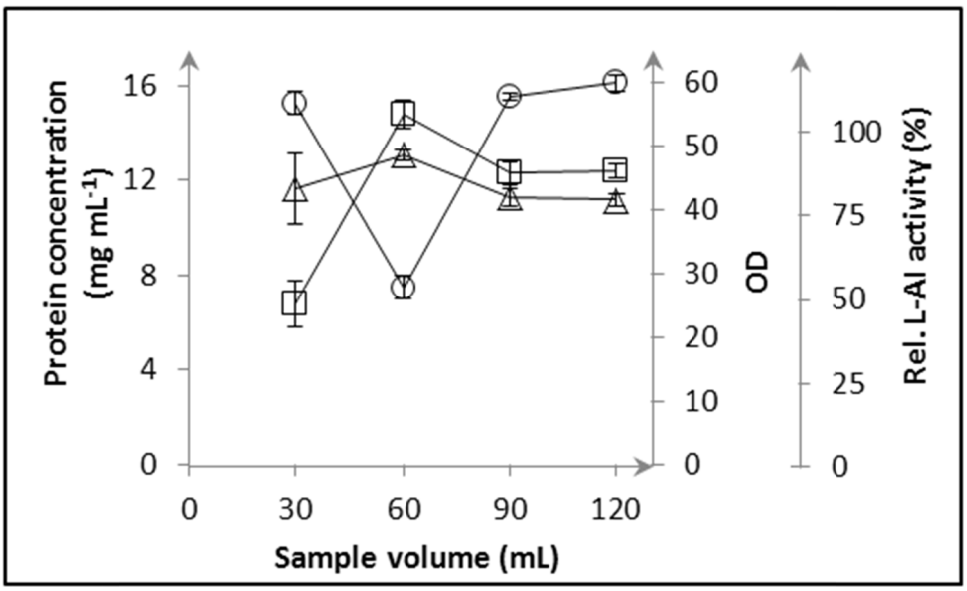

Figure 4. Effect of sample volume on the protein concentration (□) and relative L-AI activity (Δ) of the crude extract and OD of the cell debris (○)

A sample volume of 30.0 mL resulted in a low protein concentration and high OD of the cell debris which can be correlated to a high amount of eddies and shockwaves per volume unit, resulting in inactivation of the L-AI enzyme. The highest protein concentration and relative L-AI activity was found for a sample volume of 60.0 mL, namely 14.8 ± 0.6 mg mL^{-1} and 92.5 ± 0.9%, respectively. Further increase of the volume resulted in a lower protein concentration and relative L-AI activity which can be explained by a decrease of the amount of eddies and shockwaves per volume unit resulting in a lower degree of cell disruption. Thus, a sample volume higher or lower than 60.0 mL led to a lower relative L-AI activity indicating that a sample volume of 60.0 mL is preferred for sonication of the cells.

Summarized, the results show that sonication is an effective method for isolation of L-AI from *Geobacillus stearothermophilus* expressed in *E. coli*. The experimental data of all investigated parameters show that highest isolation of L-AI enzyme was obtained at 120 W after 5 minutes sonication, at a duty cycle of 50% and sample volume of 60.0 mL.

3.5 Screening of Chemical Reagents for Cell Disruption

Cell disruption can also be achieved by a broad range of chemical reagents (Falconer et al., 1997; Geciova et al., 2002; Harrison, 1991; Middelberg, 1995; Naglak & Wang, 1990; Seddon et al., 2004; Zhao & Yu, 2001). In this study, different chemical reagents were screened for isolation of the L-AI enzyme from *E. coli*. More specifically, the detergents triton X-100 and tween80, urea as chaotrope and the solvent isopropanol were studied. The efficiency of these chemical reagents were evaluated by measuring the protein concentration and relative L-AI activity in the crude extract and results are presented in Table 1.

Table 1. Effect of 16.5 mM triton X-100, 8.17 mM tween80, 2.00 M urea and 1.31 M isopropanol at 25°C on the protein concentration and relative L-AI activity of the crude extract

Chemical reagent	Protein conc. (mg mL^{-1})	Relative L-AI activity (%)
Triton X-100	15.1 ± 1.5	95.6 ± 1.2
Tween80	11.1 ± 0.3	50.3 ± 0.6
Urea	7.2 ± 0.4	33.7 ± 0.3
Isopropanol	3.5 ± 0.4	27.6 ± 2.4

The detergent triton X-100 was most effective for cell disruption of *E. coli* cells compared to tween80, urea and isopropanol. These findings are in accordance with the data of Zhao et al. (2001). These authors investigated the release of intracellular proteins from *E. coli* and found an enzyme release of 61.5 and 25.6% after treatment with triton X-100 and tween80, respectively. During our research, still a higher relative enzyme activity was observed after sonication. A high protein concentration, more specifically 15.1 ± 1.5 mg mL^{-1}, was found after treatment of washed pellet of the WT enzyme with 16.5 mM triton X-100. Furthermore, a relative L-AI activity of 95.6 ± 1.2% was noticed, which is a slightly higher activity compared to the relative L-AI activity which was reached after sonication of the cells (92.5 ± 0.9%). Thus, chemical lysis with triton X-100 resulted in a gain of 3% in relative L-AI activity compared to sonication. However, the application of chemical reagents will inevitably lead to an increase in costs due to the cost price of the chemical reagents themself, additional purification steps and more complex waste water treatment steps. Treatment of *E. coli* cells with 8.17 mM tween80, 2.00 M urea and 1.31 M isopropanol at 25°C resulted in a poor cell disruption compared to triton X-100 and sonication. Furthermore, also the isolation of WT_i enzyme was examined. 50.0 mL washed pellet of the WT_i enzyme was incubated with 16.5 mM triton X-100 during 17 hours at 25°C. The relative L-AI activity and protein concentration were 13.0 ± 0.2 g L^{-1} and 84.0 ± 0.5%, respectively. The L-AI activity and protein concentration were lower compared with chemical treatment of washed pellet of the WT enzyme (see Table 1) although a similar L-AI activity was reached at the end of the fermentation. In order to improve the cell lysis, the effect of treatment time and triton X-100

concentration were further evaluated.

3.6 Effect of Treatment Time and Triton X-100 Concentration

In order to enhance the isolation of the L-AI enzyme (WT_i enzyme), the effect of treatment time on chemical cell disruption was thoroughly examined. 50.0 mL washed pellet of the WT_i enzyme was incubated with 16.5 mM triton X-100 for 6, 17 and 28 hours. Furthermore, the effect of triton X-100 concentration on isolation of L-AI enzyme was evaluated by incubating 50.0 mL washed pellet of the WT_i enzyme with 16.5, 33.1 and 49.6 mM triton X-100 during 17 hours at 25°C (see Table 2).

Table 2. Effect of treatment time and triton X-100 concentration at 25°C on the protein concentration and relative L-AI activity of the crude extract

		Protein conc. (mg mL^{-1})	Relative L-AI activity (%)
Treatment time (h)	6	13.3 ± 0.6	79.0 ± 1.2
	17	13.0 ± 0.2	84.0 ± 0.5
	28	14.4 ± 0.8	85.7 ± 1.3
Triton X-100 concentration (mM)	16.5	13.0 ± 0.2	84.0 ± 0.5
	33.1	13.3 ± 0.1	83.7 ± 1.1
	49.6	13.3 ± 0.2	85.9 ± 0.9

Increasing the treatment time from 6 to 28 hours resulted in an increase in protein concentration from 13.3 ± 0.6 to 14.4 ± 0.8 mg mL^{-1} and in relative L-AI activity from 79.0 ± 1.2 to 85.7 ± 1.3%, respectively. Increasing the triton X-100 concentration from 16.5 to 49.6 mM had no pronounced effect on the protein concentration and only a small increase in relative L-AI activity was noticed, from 84.0 ± 0.5 to 85.9 ± 0.9%, respectively. In the context of the further purification steps in order to implement the enzyme in the biochemical production process of D-tagatose, a detergent concentration of 16.5 mM triton X-100 for 28 hours is preferentially applied. A higher concentration of triton X-100 only resulted in a limited increase in enzyme release, while a higher chemical reagent concentration will negatively influence the cost price of the enzyme. The isolation of WT_i enzyme was however still less efficient compared to the WT enzyme, viz. a relative L-AI activity of 85.7 ± 1.3 and 95.6 ± 1.2% was found, respectively. The host organism for both enzymes is *E. coli* and similar activities were present in the pellet before cell lysis. However, differences are present in the fermentation protocols e.g. fermentation time and temperature. These parameters can have an effect on cell morphology and consequently also on the efficiency of cell disruption. For this reason, a combination of chemical lysis and sonication was examined in order to attempt to increase the isolation of WT_i enzyme.

3.7 Combination of Sonication With Chemical Cell Disruption

Within these experiments, it was investigated whether isolation of WT_i enzyme can be enhanced by a combined disruption approach, e.g. sonication and chemical cell disruption with triton X-100. The order of applying different techniques can also play a major role. Sonication was followed by chemical cell lysis and vice versa. Sonication was performed with 60.0 mL sample volume at 120 W for 5 minutes with a duty cycle of 50%. Chemical cell lysis was performed in the presence of 16.5 mM triton X-100 at 25°C during a short incubation period of 6 hours. In Table 3, the mean value of the protein concentration and relative L-AI activity after cell disruption and the standard deviations are presented. Results of the combined approaches are compared to the results of the individual disruption techniques.

Table 3. Combination of sonication and chemical cell disruption for isolation of L-AI from *Geobacillus stearothermophilus*

Disruption method	Protein conc. (mg mL^{-1})	Relative L-AI activity (%)
Sonication	12.9 ± 0.8	79.3 ± 1.5
16.5 mM triton X-100	13.3 ± 0.6	79.0 ± 1.2
Sonication + 16.5 mM triton X-100	15.1 ± 0.1	78.8 ± 1.1
16.5 mM triton X-100 + sonication	16.3 ± 0.3	89.6 ± 0.3

Results in Table 3 show that a combined disruption approach enhanced the protein concentration in the crude extract. An increase in relative L-AI activity is, however, only noticed when chemical lysis was performed prior to sonication resulting in an L-AI activity of 89.6 ± 0.3% compared to 79.3 ± 1.5 and 79.0 ± 1.2% for the individual approaches, e.g., sonication and chemical cell lysis with triton X-100, respectively. The data also illustrated that sonication and chemical lysis with 16.5 mM triton X-100 during 6 hours provided a similar L-AI activity. Sonication of washed pellet of the WT_i enzyme led to a smaller protein concentration and relative L-AI activity in the crude extract compared to sonication of WT washed pellet (see Figure 1 and Table 3). A protein concentration of 12.9 ± 0.8 and 14.8 ± 0.6 mg mL^{-1} and relative L-AI activity of 79.3 ± 1.5 and 92.5 ± 0.9% was found for the WT_i washed pellet and the WT washed pellet, respectively. Through combination of a prior chemical lysis and sonication with washed pellet of the WT_i enzyme, an increased L-AI activity was reached of 89.6 ± 0.3 %. Thus, isolation of the L-AI enzyme (WT_i enzyme) can be improved by a combined disruption approach of a prior chemical lysis with triton X-100 followed by sonication. However, this implies a more complex disruption system, and hence a higher cost price. So, a compromise must be taken into account between the gain in enzyme release and corresponding cost price of the disruption approach.

In this study, different methods were investigated for isolation of *Geobacillus stearothermophilus* L-AI, intracellularly expressed in *E. coli,* in the context of the enzymatic

production of D-tagatose. Several parameters which can influence the efficiency of sonication for isolation of the L-AI enzyme were investigated with washed pellet of the WT enzyme, namely sonication time, duty cycle, power and sample volume. For each parameter, a compromise between maximal cell disruption and minimal enzyme inactivation should be made. The conditions for isolation of L-AI are respectively defined as 5 minutes sonication at 120 W with a duty cycle of 50% and sample volume of 60.0 mL. Under these circumstances, a relative L-AI activity in the crude extract was found of 92.5 ± 0.9%. Next to sonication, different chemicals, e.g., triton X-100, tween80, isopropanol and urea were screened for their efficiency in disrupting the *E. coli* cell wall. Treatment of washed pellet of the WT enzyme with 16.5 mM of the detergent triton X-100 at 25°C for 17 hours gave even better results compared to sonication, namely, a relative L-AI activity in the crude extract of 95.6 ± 1.2%. Washed pellet of the WT_i enzyme was also treated with 16.5 mM triton X-100 at 25°C for 17 hours. However, a lower L-AI activity was obtained compared to chemical cell lysis of washed pellet of WT enzyme, namely 84.0 ± 0.5. Hence, combined disruption approaches for isolation of L-AI enzyme were investigated. A prior chemical lysis followed by a sonication step, led to an increased relative L-AI activity compared to the individual approaches, e.g. chemical lysis and sonication. The relative L-AI activities were 89.6 ± 0.3%, 79.0 ± 1.2% and 79.3 ± 1.5%, respectively. The results indicate that sonication and chemical cell disruption with triton X-100 are effective methods for isolation of the L-AI enzyme from *E. coli* for further implementation in the D-tagatose production process. Furthermore, the release of recombinant proteins can further be enhanced by a combined disruption approach.

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