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PII: S0960-8524(17)31517-1
DOI: http://dx.doi.org/10.1016/j.biortech.2017.08.203
Reference: BITE 18812

To appear in: *Bioresource Technology*

Received Date: 12 July 2017
Revised Date: 29 August 2017
Accepted Date: 30 August 2017

Please cite this article as: Hohagen, H., Schwarz, D., Schenk, G., Guddat, L.W., Schieder, D., Carsten, J., Sieber, V., Deacidification of Grass Silage Press Juice by Continuous Production of Acetoin from its Lactate via an Immobilized Enzymatic Reaction Cascade, *Bioresource Technology* (2017), doi: http://dx.doi.org/10.1016/j.biortech.2017.08.203

Deacidification of Grass Silage Press Juice by Continuous Production of Acetoin from its Lactate via an Immobilized Enzymatic Reaction Cascade

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Abstract

An immobilized enzymatic reaction cascade was designed and optimized for the deacidification of grass silage press juice (SPJ), thus facilitating the production of bio-based chemicals. The cascade involves a three-step process using four enzymes immobilized in a Ca-alginate gel and uses lactic acid to form acetoin, a value-added product. The reaction is performed with a continuous, pH-dependent substrate feed under oxygenation. With titrated lactic acid yields of up to 91% and reaction times of ca. 6 h was achieved. Using SPJ as titrant yields of 49% were obtained within 6 h. In this deacidification process, with acetoin one value-added bio-based chemical is produced while simultaneously the remaining press juice can be used in applications that require a higher pH. Such, this system can be applied in

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a multi-product biorefinery concept to take full advantage of nutrient-rich SPJ, which is a widely available and easily storable renewable resource.

Keywords

Enzymatic reaction cascade, Grass silage press juice, Enzyme immobilization, Biocatalysis, Biorefinery

1 Introduction

Biorefineries offer a promising solution to sustainably meet the increasing demand for fuels and chemicals. They combine multi-product and integrative concepts, which allow the complete utilization of biological resources, making them more economical (Cherubini, 2010). Issues of "food or fuel discussions" can be avoided by choosing the appropriate raw material. A possible resource with little to no cannibalization of food feedstocks is lignocellulosic biomass from grass (Huang et al., 2008). Such graminaceous plants are widely distributed, covering 40.5% of the world's land mass. The European Union has continued to preserve existing grassland ecosystems to comply with environmental standards. Along with the decline in agricultural feed consumption, surpluses of grass biomass with an annual yield of 8 t/ha are being generated. This released grass biomass can be used as an additional feedstock for alternative conversion pathways, such as new biotechnological applications (European-Commission, 2013; Mandl, 2010). Additionally, this excess will be a possible target resource for new biotechnological applications, since the release of unused biomass from the permanent grassland of the European temperate grassland regions will rise and the amount of dairy cow husbandry and cattle breeding (the main consumers of grassland) is expected to drop (Kamm et al., 2008). Grass biomass is a perishable resource, and for its

conservation, ensilaging has been used for centuries. During this process, anaerobic bacteria (mainly lactic acid bacteria) convert easily accessible carbohydrates to short-chain carboxylic acids, predominantly acetic and lactic acids. With the production of these acids, the pH of the wet raw materials decreases, preventing the growth of spoilage microbes. After an initial solid–liquid separation, the soluble components of the press juice are directly available for biotechnological applications. A breakdown of the fibrous solid silage matter, mainly lignocellulose, further enhances the potential for applications (Schwarz et al., 2016; Xiu & Shahbazi, 2015). In this process, two resource streams (solid and liquid) are created, and if necessary, the solid phase can still be used as feedstock since the fibrous matter is the main nutrient source for ruminants. A multi-product biorefinery system would integrate these processes, so that all resource streams can be used to their full potential (Ecker et al., 2012; Kamm et al., 2008; Kromus et al., 2004; Mandl, 2010).

By pressing the moist grass silage, a liquid fraction is generated, termed SPJ. Due to their natural origin and different production methods, various SPJs may vary distinctly in some of their content, while staying remarkably similar in others. The carbohydrate concentration, for example, can vary from 6.8 g/L (Kromus et al., 2004) to 26.9 g/L (Thang & Novalin, 2008). In contrast, the overall protein concentration is rather conserved, ranging from 25 g/L (Ecker et al., 2012) to 29 g/L (Kamm et al., 2008; Kromus et al., 2004). Lactic acid is the most abundant component in SPJs, with concentrations ranging from 30.5 g/L (Kromus et al., 2004) to 37.5 g/L (Kamm et al., 2008; Thang & Novalin, 2008), resulting in pH values close to pH 4. The high concentrations of various organic acids and carbohydrates, proteins and amino acids, metal ions and vitamins make SPJs a very nutrient-rich resource, and consequently they have already been trialed as a source for bacterial fermentation. Previous studies successfully used SPJs for polyhydroxybutyrate (PHB) production (Cerrone et al.,

2015) and ethanol fermentations (Sieker et al., 2011). However, SPJ remains a challenging medium for fermentative processes because the low pH caused by the high concentration of carboxylic acids inhibits bacterial growth. The selective conversion of carboxylic acids, accompanied by a rise in pH means, may provide the basis for enhanced fermentative applications. However, since it is difficult to remove lactate or acetate from aqueous solutions, a different approach needs to be established. Enzymes that specifically catalyze the transformation of carboxylic acids into non-acidic compounds can release SPJ in a form then be applied to downstream fermentations as a nutrient source. An efficient system needs to be developed whereby the pH of SPJ is raised without adding large amounts of base and where the nutrients from SPJ are preserved for subsequent biotechnological processes. A possible system employs a cell-free enzymatic reaction cascade that acts specifically on the carboxylic acid under mild reaction conditions, and in so doing leaving other reactants intact. Cell-free enzymatic reaction cascades are processes in which enzymes from various sources are combined to form *in vitro* pathways. These pathways can be natural or non-natural as long as the applied enzymes function together. Several reports have been published on the development of cell-free enzymatic cascades involving a range of substrates for the production of biofuels, electricity, biocommodities and high-value chemicals (Beer et al., 2017; Guterl & Sieber, 2013; Hodgman & Jewett, 2012; Jandt et al., 2013; Zhang, 2010) A major advantage of cell-free systems is that they circumvent complex genetic modifications of microorganisms by producing the required enzymes in a separate process. Additionally, enzymes from all domains of life, and individually engineered to meet the requirements of the cascade, can be combined to form an optimized pathway (Heinzelman et al., 2009; Lutz, 2010; Steffler et al., 2013). For the development of a cell-free system, that selectively deacidifies SPJ by reducing the amount of organic acids while producing a value-added

compound, lactic acid is a suitable target substrate. Lactic acid is highly abundant in SPJ, and its pK_a of 3.86 is lower than that of acetic acid (4.76). The contribution of lactic acid to the low pH is therefore considerable, and with a reduction in the overall amount of lactic acid, a significant rise in pH can be expected.

There are two criteria that need to be considered for the development of this specific reaction cascade. Firstly, the product needs to be non-acidic and the cascade needs to be as simple as possible. Secondly, a co-substrate (*e.g.* NAD^+) dependence would increase the complexity and vulnerability of the system. Thus, a cofactor-independent reaction cascade is preferable. Acetoin (3-hydroxybutanone) is an ideal target product for this study; it has no effect on pH and can be converted from lactic acid in only three reaction steps. The starting reaction, *i.e.* the conversion of lactic acid to pyruvate, a major intermediate in both metabolism and in previously established successful cell-free reaction cascades (Guterl et al., 2012), can be catalyzed by two distinct enzymes, (i) lactate dehydrogenase (LDH), which converts lactate to pyruvate with NAD^+ as a co-substrate, or (ii) lactate oxidase (LOX), which requires molecular oxygen as a co-substrate. LDH is well characterized, easy to handle and commercially available. However, its major drawback is its requirement for the co-substrate NAD^+, which must be added stoichiometrically or regenerated by additional enzymes. Furthermore, nicotinamide cofactors are not very stable under non-neutral conditions (Nowak et al., 2015). In contrast, LOX converts lactate to pyruvate with oxygen acting as the ultimate electron acceptor. This reaction is FMN-dependent, and since FMN is tightly bound to the enzyme (Holt et al., 2016), it does not require stoichiometric addition or a co-substrate recycling system. The disadvantage of using LOX is the release of hydrogen peroxide, which needs to be removed from the system to prevent its oxidizing capability

from reducing enzyme activity (Finnegan et al., 2010). In our reaction setup, we used LOX but also added catalase to remove the hydrogen peroxide.

Following the formation of pyruvate two additional enzymes are needed to produce acetoin: acetolactate synthase (ALS) and acetolactate decarboxylase (ALDC). We previously demonstrated that ALS can be applied in cell-free reaction cascades to produce isobutanol (Guterl et al., 2012). By using Mg^{2+} and thiamine pyrophosphate (TPP), both co-factors of ALS, 2-acetolactate is produced, which subsequently is decarboxylated by ALDC (Wiegeshoff & Marahiel, 2007). ALDC is a well-studied enzyme applied on a large scale during brewing processes, where it prevents the formation of diacetyl, an undesirable flavoring agent (Dulieu et al., 2000; Marlow et al., 2013). Using this cascade, two moles of pyruvate are converted into one mole of acetoin. Over recent years, interest in acetoin has risen because of its wide range of applications. It is not only a naturally occurring non-toxic flavoring agent but can also be used as a biofuel or fuel additive, or as a precursor for plastics and epoxy resins (Liu et al., 2011; Sun et al., 2012; Xiao & Xu, 2007).

SPJ is a heterogeneous mixture and consists of various chemically and physiologically active components. Consequently, it has been difficult to use SPJ as a substrate for conventional fermentations. Here, we show that SPJ can be readily deacidified with the cell-free enzymatic reaction cascade shown here to make it a suitable substrate for use in fermentations while being simultaneously used as a substrate source to produce value-added chemicals.

2 Materials and Methods

2.1 Chemicals and Materials

All chemicals were, unless otherwise stated, purchased in analytical grade from Sigma-Aldrich (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Serva Electrophoresis (Heidelberg, Germany), and Merck (Darmstadt, Germany). Low-viscosity Alginate was purchased from Sigma-Aldrich (A0682). Restriction enzymes Phusion®high-fidelity DNA polymerase and T4 ligase were purchased from New England Biolabs (Frankfurt, Germany). Taq polymerase was obtained from Rapidozym (Berlin, Germany). Oligonucleotides were purchased from Thermo Scientific (Waltham, Massachusetts, USA). Lactate oxidase (LOX) and catalase (CAT) were purchased from Sigma-Aldrich.

2.2 Strains and Plasmids

In this study, the following strains were used: *Escherichia coli* HMS (DE3), *E.coli* BL21 (DE3), and *Bacillus licheniformis* DSMZ 8785. The *E. coli* codon-optimized *alsS* gene was synthesized by Geneart (Regensburg, Germany) using the published gene sequence (GenBank CAB07802.1). The acetolactate decarboxylase gene from *B. licheniformis* was amplified by PCR with genomic DNA as a PCR template. Based on the published protein sequence (GenBank WP_043926532), the following primers were designed: Fw-ALDC-*B.lich* ATATATCAT**ATG**AAAAGTGCAAGCAAACAAAAAATAATTCAG and Rw-ALDC-B.lich ATATATCTCGAGTTATTCAGGGCTTCCTTCAGTTGTTTC (the recognition sites for the restriction enzymes are underlined and the start codon is in bold). The PCR product was digested with *NdeI* and *XhoI* and cloned into pET28a (Novagen). For plasmid synthesis *E. coli* HMS (DE3) was used, and for expression *E. coli* BL21 (DE3).

Butanediol dehydrogenase (BDDH) from *B. cereus* (GenBank WP_000645824.1) was synthesized and cloned into pET28a by GenScript (Piscataway, USA). The sequence was codon-optimized for the expression in *E. coli*. All vectors used contained N-terminal His-tags.

2.3 Enzyme Expression and Purification

Protein expression was performed in *E. coli* BL21 (DE3) using LB-media with 100 µg/mL kanamycin. After growth to an OD of 0.6 at 37 °C, 1 mM IPTG was used to induce expression and the temperature was lowered to 16 °C. After 24 h, cells were harvested and disrupted with a Basic-Z Cell Disruptor (Constant Systems, Northants, UK). Cell debris was removed by centrifugation at 35,000 g for 30 min, and the protein was purified by a Ni-NTI column and subsequently desalting using an ÄKTA UPC-900 FPLC-system (GE Healthcare, Freiburg, Germany), equipped with HisTrap FF and HiPrep 26/10 Desalting-columns (GE Healthcare, Freiburg, Germany). For resuspension, the buffer used was 100 mM HEPES (pH 7) with 20 mM imidazole and 500 mM NaCl. Elution was achieved with 500 mM of imidazole. After desalting with 100 mM HEPES (pH 7), the enzymes were stored at -80 °C.

2.4 Enzyme Assays

All spectrophotometric measurements were performed using a Mulitskan or Varioscan spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). All reactions were performed under standard conditions at 40 °C in 50 mM MOPS (pH 6.5), 2.5 mM $MgCl_2$, and 0.1 mM TPP. One unit of enzyme activity was defined as the amount of enzyme needed to convert 1 µmol of substrate per minute. ALS activity was determined by monitoring the decrease in absorbance of pyruvate (50 mM) at 320 nm. The calibration was performed with an external standard. BDDH activity was measured by monitoring NADH

reduction under standard conditions at 340 nm ($\varepsilon_{(NADH)}$ = 6200 L/(mol·cm)) using 50 mM acetoin and 0.25 mM NADH.

For measuring ALDC activity its substrate 2-acetolactate was produced by incubating 200 mM pyruvate and 10 U of ALS for 20 min under standard conditions. Subsequently, ALS was removed by filtration (10 kDa MWCO, modified PES; VWR, Darmstadt, Germany). The 2-acetolactate solution was always freshly prepared and stored on ice for no longer than 1 h. ALDC activity was determined by measuring the concentration of acetoin with GC (see section 2.8) produced after 2 min under standard conditions.

For determining the optimal reaction conditions and ratio of all relevant enzymes, NADH depletion was monitored at 340 nm with excess amounts of BDDH. Experiments were performed in 96-well plates with a total volume of 200 µL per reaction. Enzyme concentrations were varied for LOX, ALS and ALDC. CAT and BDDH were kept constant at 50 and 5.9 U/mL, respectively. The reaction was performed at 40 °C with 50 mM lactic acid and 1 mM NADH in 100 mM MOPS at a pH of 6.5.

2.5 Enzyme Immobilization

2.5.1 Cross-linked Enzyme Aggregates

Enzyme aggregates were generated from purified enzymes using the following concentrations: LOX 10 mg/mL, ALS 7.4 mg/mL, ALDC 2.6 mg/mL, CAT 10 mg/mL and BSA 10 mg/mL; this represents the experimentally determined optimal ratio of the individual components. The protein mixture was precipitated with an equal volume of ice-cold saturated ammonium sulfate solution while being continuously stirred on ice for 15 min. After precipitation, 75 mM glutaraldehyde (final concentration) was added and continuously

mixed while being kept on ice for 2 h. The aggregates were collected via centrifugation and washed three times with reaction buffer (see section 2.4).

2.5.2 Enzyme Encapsulation

For encapsulation of the enzymatic reaction cascade, a 5% Na-alginate solution was prepared. To homogeneously dissolve Na-alginate, the solution was heated to 60 °C. Air bubbles were removed by allowing the solution to rest for at least 2 h. Then, 5 mL of 5% Na-alginate was mixed with 1.25 mL of enzyme solution containing LOX (0.3 mg/mL), ALS (0.57 mg/mL), ALDC (0.044 mg/mL) and CAT (1 mg/mL) with a final volume 6.25 mL. This was added to a 0.2 M $CaCl_2$ bath using a syringe with an inner diameter of 0.4 mm. The formed alginate beads were hardened for at least 1.5 h, and then stored in reaction buffer containing 0.1 mM TPP and 2.5 mM $MgCl_2$.

2.6 Preparation of Grass Silage Press Juice

The SPJ fraction investigated in this study was provided as described earlier by Schwarz et al. (2016). After the mechanical separation step of the ensiled moist biomass, SPJ was filtered through a 250 µm-filter bag (Schwegmann-Filtrations-Technik GmbH, Grafschaft-Gelsdorf, Germany) to remove coarse particles. The dark-brown liquid was stored at 4 °C in 10 L containers until further use. Prior to the enzyme cascade experiments, further clarification of the liquid phase was successively performed by centrifugation (12,000 g for 30 min), followed by filtration (retention range 5-8 µm, Type 13A Cellulose; Carl Roth GmbH, Karlsruhe, Germany), and another centrifugation (35,000°g for 30°min).

2.7 Acetoin Synthesis

For the synthesis of acetoin, 30 mL of a 50 mM lactic acid solution was prepared (Carl Roth, Karlsruhe, Germany). When using SPJ as substrate, a dilution had to be prepared meaning, that SPJ was diluted to have an equivalent amount of lactic acid. All reactions contained 0.1 mM TPP and 2.5 mM $MgCl_2$ and were aerated with 100 % oxygen at a flow rate of 10 mL/min. The reaction was run at pH 6.5 and 40 °C in 50 mM MOPS. Titrations were carried out with 100 mM HCl, 500 mM lactic acid, or undiluted SPJ using an automatic titrator (TitroLine 7000, SI Analytics, Weilheim, Germany). The reaction was started by adding the enzymes, either soluble or immobilized, to the reaction vessel. For reactions with soluble enzymes, LOX, ALS, ALDC, and CAT were added at concentrations of 50 µg/mL, 94.5 µg/mL, 7.3 µg/mL and 166.7 µg/mL, respectively, corresponding to 30, 17.86, 55 and 10000 U. These concentrations represent the experimentally determined optimal amounts for each enzyme (see below). Reactions with immobilized enzymes were carried out using alginate beads obtained by immobilizing all enzymes in one batch (see section 2.5). Samples were taken after certain time points and prepared for high-performance liquid chromatography (HPLC) analysis via filtration.

2.8 GC-FID Analysis

Acetoin concentrations were determined using a Thermo Scientific Trace GC Ultra, equipped with a flame ionization detector (FID) and an autosampler (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Separation was performed using a StabilWax column (30 m, 0.25 mm internal diameter, 0.25 mm film thickness; Restek, Bellefonte, USA), whereby helium (1 mL/min) was used as the carrier gas. The oven temperature was programmed to be held at 50 °C for 5 min and then raised by 50 °C/min to 220° C. The detector was kept at 250 °C and the injection was performed with 10 µL in split mode with 13 mL/min. For acetoin

quantification, an external standard was applied. Acetoin standards and samples were extracted with ethyl acetate in a ratio of 1:1. The detection limit was determined to be 0.5 mM.

2.9 HPLC

The concentrations of organic acids and formation products in the aqueous solutions were quantified using an HPLC system (Dionex®, Sunnyvale, CA, USA) equipped with a Rezex ROA-H^+ column (Phenomenex®, Torrance, CA, USA), a refractive index detector (RI 101, Shodex, Tokyo, Japan), and a PDA detector (210/278 nm, Dionex®, Sunnyvale, CA, USA). The mobile phase (sulfuric acid, 2.5 mM) was set to a flow rate of 0.5 mL/min at an oven temperature of 70 °C. Prior to measurement, all samples were filtered through a 0.2-µm PVDF filter (Restek GmbH, Bad Homburg, Germany). Qualitative analyses and quantitative calculations of each compound were referred to an external standard. Detection limits were determined to be 0.3 mM for lactic acid and acetic acid, and 43 µM for pyruvate.

3 Results and Discussion

3.1 Determination of Cascade Enzyme Composition

A major goal for the development of cell-free reaction cascades is to achieve a constant reaction flow. An accumulation of any intermediate is unwanted and could lead to undesirable side reactions (Ye et al., 2012). It is therefore crucial that each enzyme and its corresponding reactions are in balance with each other according to their activity. Jaturapaktrarak *et al.* reported a method of performing a flux analysis (Jaturapaktrarak et al., 2014). Here, this method was adopted and altered to determine the exact enzyme units needed to balance the reaction. With the use of a reporter enzyme it was possible to couple the cascade to an NADH-dependent reaction. This enzyme, BDDH from *B. cereus*, converts acetoin and NADH to 2,3-butanediol and NAD^+, which can be monitored spectrophotometrically at 340 nm. It was then possible to rapidly determine the concentrations of each enzyme that is required, maintaining a constant flux through the system, and thus a balanced reaction. This was achieved by keeping the concentrations of all enzymes but one constant and varying the concentration of the remaining one until the highest possible flux was reached. This analysis was performed using an excess amount of BDDH. A ratio of 1 : 0.6 : 1.8 for LOX : ALS : ALDC, respectively, was determined as optimal.

3.2 Utilization of Lactic Acid for the Production of Acetoin

In order to optimally aerate and titrate the system, the reaction cascade was scaled up to run in a vessel with a 30 mL reaction volume. Titrations were carried out with 0.1 M HCl, and the first experiment was performed with 10, 5.9 and 18.3 U of LOX, ALS, and

ALDC, respectively, their optimal concentrations (*vide supra*), and 1000 U of CAT to facilitate fast removal of hydrogen peroxide. The results were unsatisfactory since only 10 % of the added lactic acid was converted to acetoin, and the entire reaction stopped after 15 min. The same experiment was then repeated using twice the amount of each enzyme (20, 11.8, 36.6 and 1000 U, respectively, for LOX, ALS, ALDC and CAT), thus providing information about the stability of these enzymes under reaction conditions. This two-fold increase led to a 25% conversion of lactic acid to acetoin and a more stable process, being active for more than 30 min. These results were promising, but the process still could not achieve complete conversion and the lack of stability remained a major concern.

Another issue was the rise in the concentration of acetic acid, which is likely to be due to an incomplete reduction of H_2O_2, which may influence enzyme stability. The added concentration of CAT was too low to reduce all H_2O_2, thus leading to a significant increase in its concentration. Since CAT can be obtained cheaply and in large quantities, and has a very high specific activity of 2000 U/mg, a 10-fold increase in activity was easily possible. This ensured a very low H_2O_2 concentration, which increased protein stability and suppressed the formation of acetic acid.

Based on the insight gained from the previous experiments a process containing the following enzyme Units was setup: 30, 18, 55 and 10,000 U of LOX, ALS, ALDC and CAT, respectively. The results are summarized in Fig. 1 and show a linear decrease in lactic acid within the first 30 min. Since titration is performed only when the pH rises, we assume that the addition of hydrochloric acid to the system directly correlates to the rate of substrate conversion. These results suggest that a complete loss of enzyme

activity can be seen after 2 h. To test this hypothesis, an equal amount as at the beginning of fresh enzyme was added to the system, which resulted in the immediate resumption of substrate conversion, as shown by the onset of titration. During the first 2 h, 38% of the lactic acid was converted to acetoin. The second addition of the enzyme increased the yield to 74%. Therefore, we concluded that the main reason for the conversion rate to significantly slow down after 30 min (and completely fade after 2 h) was the loss of enzyme activity. LOX needs oxygen to be active; thus, aeration is essential to maintain stable reaction rates. Thus, to keep oxygen levels high within the reaction vessel, it was aerated with 100% oxygen at a flow rate of 10 mL/min. However, this aeration resulted in protein precipitation. Several attempts to decrease the effects of aeration in the setup with free enzymes were unsuccessful.

3.3 Immobilization of the Reaction Cascade

Enzyme immobilization may provide an avenue to overcome enzyme destabilization due to oxygen aeration. In order to achieve the immobilization of all required enzymes in one compartment two methods were chosen and compared: cross-linking and encapsulation within alginate beads. The cross-linking method is based on the reactivity of primary amine residues on the protein surface. These amines, deployed by lysine residues, are cross-linked using glutaraldehyde (Scism & Bachmann, 2010). During this process, the enzymes form small particles and can be utilized as heterogeneous catalysts.

Encapsulation was selected because it is possible that the large surface area of oxygen bubbles inside the reaction solution causes denaturation of the proteins, and hence a

protective layer around the enzymes could improve the stability of the reaction cascade. A major disadvantage of this method is, however, the introduction of a diffusion barrier for substrates and intermediates. Here, a well-described alginate entrapment method (Haider & Husain, 2007) was chosen to probe the usefulness of encapsulation. The effect of the two immobilization techniques on the reaction cascade, in comparison to the free enzyme approach, is shown in Fig 2. While the activity of the cascade that uses enzymes in solution disappeared after 2 h, both immobilized reaction cascades are active after 6 h, albeit with a decreased rate. For normalization purposes, all values in Fig. 2 are given in µmol acetoin formed instead of concentrations since the total reaction volume changes during the rate measurements due to the addition of the acid in the titration. Ultimately, the alginate encapsulation method was chosen as the immobilization technique for subsequent experiments, not only due to the higher activity and conversion rate when compared to the cross-linking approach, but also for practical reasons. Cross-linking had major disadvantages in preparation and handling. It took in the excess of 4 h to produce a batch of ready-to-use immobilized enzymes. Additionally, it was very difficult to distribute the catalyst evenly inside the reaction vessel. Specifically, during the cascade reaction large agglomerates were formed that adhered to the reactor walls and rendered the whole process error-prone and therefore unreliable.

In contrast, encapsulation reduced the preparation time to less than 2 h and handling of the beads was simple and reproducible. Furthermore, the capsules allow easy separation of the catalysts from the reaction solution. We noted that low-viscosity alginate performed best under the established experimental conditions. Alginate with

higher viscosity dissolved quickly after application to the reactor. The major drawback of encapsulation is the introduced diffusion barrier; both the substrate and product have to diffuse in and out of the beads, and oxygen must diffuse through the alginate capsule to keep the process running. Previous reports indicated that a bead size of less than 2 mm was most effective. Smaller sizes had very little effect on the oxygen uptake rates (Chen & Huang, 1988). A second parameter that affects the diffusion rate is the alginate concentration. A higher concentration can increase the bead stability but will also decrease the diffusion rates. A 4% alginate solution provided the optimal compromise in our cascade. Beads produced with lower alginate concentrations dissolved during the reaction process, whereas those produced with higher concentrations performed less efficiently (data not shown).

3.4 Encapsulated Enzymatic Reaction Cascade for the Continuous Production of Acetoin

In order to evaluate the feasibility of the enzymatic approach for the deacidification of SPJ and the production of acetoin, a continuous substrate feed was established using a solution of lactic acid as titrant. This guaranteed that the basic pH shift was continuously neutralized, while the substrate was simultaneously fed to the reaction. A 0.5 mM solution of pure lactic acid was tested as a well-defined substrate. Fig 3 shows a complete overview of the acetoin forming process. In this experiment, the titration with lactic acid was additionally used as a direct indicator for the activity of the reaction cascade.

After approximately 30 min the acetoin concentration reached a detectable level. A steady increase in the concentration of product was measured up to 5 h. Because of the continuous pH regulation using lactic acid, its overall concentration remained constant. During the 6 h of reaction time, 91% of the added lactic acid was converted. Pyruvate reached a maximum of 4.5 mM after approximately 2 h, and then decreased to 2.7 mM. This behavior can be explained by the way pyruvate reacts with hydrogen peroxide to form acetic acid in a side reaction (Asmus et al., 2015). Acetic acid concentration was measured at several time points. Since hydrogen peroxide never showed a detectable increase in its concentration unless CAT was deliberately left out of the system, it is plausible that this side reaction occured immediately inside of the beads.

By calculating the mass balance over all measurable intermediates, we elucidated how much of the initial substrate was lost to this side reaction. After 6 h, 94% of all the introduced substrate was found within measurable and favorable compounds related to the reaction cascade, with only 6% of substrate being lost to either unfavorable or immeasurable side-product formation because of the hydrogen peroxide reaction or, additionally, the evaporation and extraction of the volatile compounds at 40 °C by aeration with oxygen.

Since full conversion was not possible in a setup of continuous substrate feed, the yield was determined by calculating the amount of fed lactic acid and produced acetoin. During the first 6 h of the reaction, 91% of the added lactic acid was converted to acetoin. With respect to the overall substrate amount within the reaction vessel (fed lactic acid plus initial quantity of lactic acid), a yield of 47.9% was calculated. A final

acetoin concentration of 24.4 mM, out of a possible 50.8 mM, was obtained and a reaction rate of 7.43 mM/h (0.65 g/L/h) during the first hour was achieved. These experiments thus demonstrated that a stable and continuous production of acetoin is possible using lactic acid as a substrate for an immobilized cell-free enzymatic reaction cascade. The reaction demonstrated negligible side reactions and appeared to be stable toward oxygenation due to the immobilization of the enzymes in Ca-alginate beads.

3.5 Lactic Acid from SPJ as a Source of Acetoin Production

While the results with pure lactic acid as a substrate of the immobilized cascade were promising, the suitability of SPJ as a substrate needed to be demonstrated. For this, we freshly prepared SPJ from a farm (agricultural area: Eastern Bavarian Low Mountain Range) in the province of Upper Palatinate, Bavaria, Germany, according to a previously reported methodology (Schwarz et al., 2016). The recovered SPJ amounted to 0.53 L/kg grass silage, with a density of 1.05 g/mL, a dry matter (DM) content of 12.2% and a pH of 4.3 (Schwarz et al., 2016). Lactic acid, with an average concentration of 35.3 g/L, was the most abundant compound, correlating to 28.7% of the DM content, followed by proteins and water-soluble carbohydrates with concentrations of 30.3 and 28.5 g/L, respectively. Acetic acid accounted for 8.1% DM, or 10.0 g/L. Furthermore, various metal ions such as potassium, calcium or sodium were detected at different concentrations.

Since SPJ is a very complex reaction medium with a multitude of different compounds, each of which might have a considerable influence on the different enzyme activities

within the reaction cascade, we expected a significant reduction in cascade performance. In the first experiment, the reaction was set up with 50 mM lactic acid and titrated with undiluted SPJ containing 378 mM of lactic acid. Fig 4 shows that the cell-free enzymatic reaction cascade could indeed utilize SPJ as a substrate. The reaction cascade ran for 6 h. By the time the reaction was stopped, 1.8 mL of SPJ was fed into the system. The overall amount of produced acetoin was 16.8 mM at a maximum rate of 6.5 mM/h (0.57 g/L/h) during the first hour. The cascade was consistently active for the 6h period. This was comparable to the results of the experiments conducted with pure lactic acid. The amounts of acetic acid shown in Fig 4 were corrected for the acetic acid introduced by the SPJ, and thus only represent the amount of acetic acid formed by the reaction cascade. This is also the reason why the lactic acid concentration decreased instead of staying constant, as observed in the previous experiments (Fig 3). Acetic acid was accumulated during the process because of the titration with SPJ, and thus the decrease in the observed pH was not solely due to lactic acid. The overall conversion yield of 49% is comparable with the results of the experiments with the pure lactic acid solution (*vide supra*). The time course for the concentration of pyruvate is also similar in the two experiments using pure lactic acid or SPJ as substrate (Fig 3, Fig 4). For the SPJ titration experiment, the mass balance accounted for 95% of all substances related to the reaction, implying that only 5% of the substrate was lost during the reaction process. This experiment shows that SPJ can be used as a titration agent to keep the reaction rate almost constant for up to at least 6 h. Encouraged by these results, we tested whether the reaction cascade would also

work with SPJ as an exclusive source of lactic acid. The relevant results are illustrated in Fig 5.

The setup was similar to that of the experiments described above. SPJ was diluted to an initial concentration of 50 mM lactic acid; the corresponding concentration of acetic acid was 21.1 mM. Additionally, the reaction was titrated using undiluted SPJ. Analysis of these experiments showed that acetoin synthesis was readily detectable. The immediate formation of acetoin indicated high activity of the enzyme cascade. Moreover, it showed that the lactic acid obtained from SPJ is a feasible source of enzymatic conversion. The mass balance calculations showed very little loss of substrate toward unfavorable side products during the conversion process. All compounds related to the reaction cascade accounted for a mass balance of 93%. When using undiluted SPJ as titrant, pyruvate concentrations remained fairly low at 0.6 mM compared to 4.5 mM when pure lactic acid was used as substrate. Surprisingly, pyruvate reached a lower steady-state concentration of 0.5 mM after 1 h when SPJ was the sole substrate for the cascade. By using pure lactic acid as the substrate, the corresponding pyruvate concentration was approximately 3 mM (Fig 3). A comparison of the rates of the reactions with either pure lactic acid or lactic acid within SPJ as substrate revealed only a 17% decrease in overall activity of the latter, although the total reaction time over which this cascade remained active was lower. After 4 h no significant further increase in acetoin concentrations could be monitored (Fig 4). The total amount of acetoin was 11.5 mM, and hence the total yield was only 37%.

A visual inspection reveals that the alginate beads adopt the dark-brown color of SPJ as the reaction proceeds. Although SPJ was filtered and centrifuged twice prior to use,

colloidal sediments remained in the liquid phase, which adhered to the beads and caused their observed color change. Additionally, these colloidal particles may also have caused the bead pores to clog, thus reducing the diffusion rate of substrates and products into and out of the beads. Clogging and the associated reduction in diffusion may indeed be the main reason for the reaction rate, yield, over time and pyruvate concentration to be lower when SPJ rather than pure lactic acid was used as substrate. Furthermore, it cannot be ruled out that the immobilized enzymes are inhibited, at least partially, by various inorganic and organic substances present in SPJ. Since it is difficult to distinguish among physical, chemical and enzymatic disturbances caused by substances found in SPJ, a detailed characterization of the SPJ mixture on the functional properties of each of the enzymes of the cascade may be necessary to further improve the overall performance of the reaction. Such characterizations are beyond the scope of the present study. However, its impact needs to be viewed in terms of the first demonstration of the capability of a cell-free encapsulated enzymatic reaction cascade to convert lactic acid from SPJ to acetoin, and in the process deacidify SPJ, thus making this source of substrate available for further use. With an improvement in enzyme immobilization, and an enhancement of enzymatic efficiency through evolution techniques, it should be possible to enhance overall processing times and yields. In combination with a highly selective extraction of acetoin, *e.g.*, by a stripping process, this reaction cascade may emerge as a potent addition to a multi-product Biorefinery (Sun et al., 2012; Xiu & Zeng, 2008).

3.6 Economical Evaluation

The aim of developing a reaction cascade, such as in this study, is its implementation in a biorefinery concept, where it may serve as part of the complete utilization of grass silage (Ecker et al., 2012; Kamm et al., 2008; Schwarz et al., 2016). An immobilized reaction cascade is simple to use, as the reaction beads can be easily applied and removed from SPJ. Furthermore, there is no need for any additional cofactor, making its application very suitable for upscaling.

The most important issue with integrating a cell-free enzymatic reaction cascade into technical processes is the high enzyme cost. A definitive enzyme cost analysis at this point is not possible since not all of the enzymes are commercially available. Current prices for ALDC and CAT are reasonably low, since they are already being produced and applied in large-scale processes. ALDC costs amount to 0.16 €/g enzyme with an activity of 2 U/mg (Murphy and Sun Limited, Nottingham UK, Brewhouse Enzymes, 2017). CAT prices are in the order of 0.1 €/g with an activity of > 50000 U/mg (Xian Nutragreenlife Biotechnology). Both examples show that enzyme costs can be low when produced on a large scale. In addition enzyme engineering can be applied for increased activity. Generally, with average enzyme costs of 0.2 €/g and average activities of 30 u/mg. Although this is not yet the case for ALS and LOX their costs reduce significantly with industrial production.

For a successful implementation of this reaction cascade into a biorefinery concept the issues of process stability need to be overcome. However, since it is easy to produce and handle this system, an economical viable application may indeed be possible.

4 Conclusion

It is possible to specifically convert lactic acid within SPJ into the value-added chemical compound acetoin, thereby deacidifying SPJ and facilitating its further use as nutrient-rich substrate in other applications. The reaction is catalyzed by a three-step process using a four-enzyme reaction cascade, which is immobilized in a Ca-alginate polymer. For a possible implementation of this process into a biorefinery concept, the process stability as well as overall yields need to be increased. But with this simple reaction setup in combination with no need for any further cofactors an implementation might be possible. E-supplementary data for this work can be found in e-version of this paper online.

Acknowledgments

This research was funded by the German Federal Ministry of Education and Research; “Next-Generation Biotechnological Processes – Biotechnology 2020+” (031A177B) and the COST action CM1303 Systems Biocatalysis. We would like to thank Daniel Bauer, Jose Guillermo Ortiz Tena, and Marius Rütering for their help in the statistical analysis and continuing support.

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6 Figure Captions

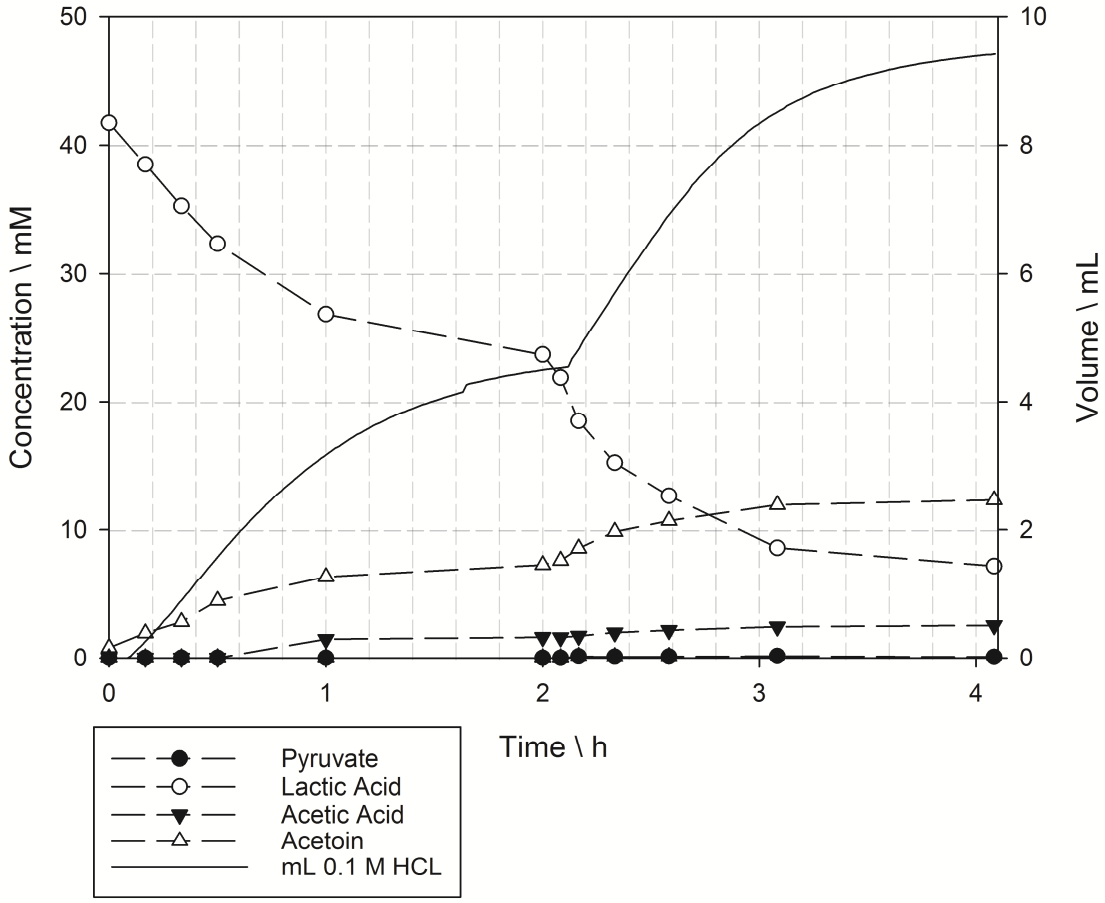
Fig. 1 Production of acetoin from lactic acid using a three-step enzymatic reaction cascade. All components were in solution and the reaction was performed at 40 °C and kept at a constant pH of 6.5. The enzyme activity units were 30, 17.86, 55 and 10000 U for LOX, ALS, ALDC and CAT, respectively. After 2.08 h fresh enzyme was added with amounts equal to those at the beginning of the experiment.

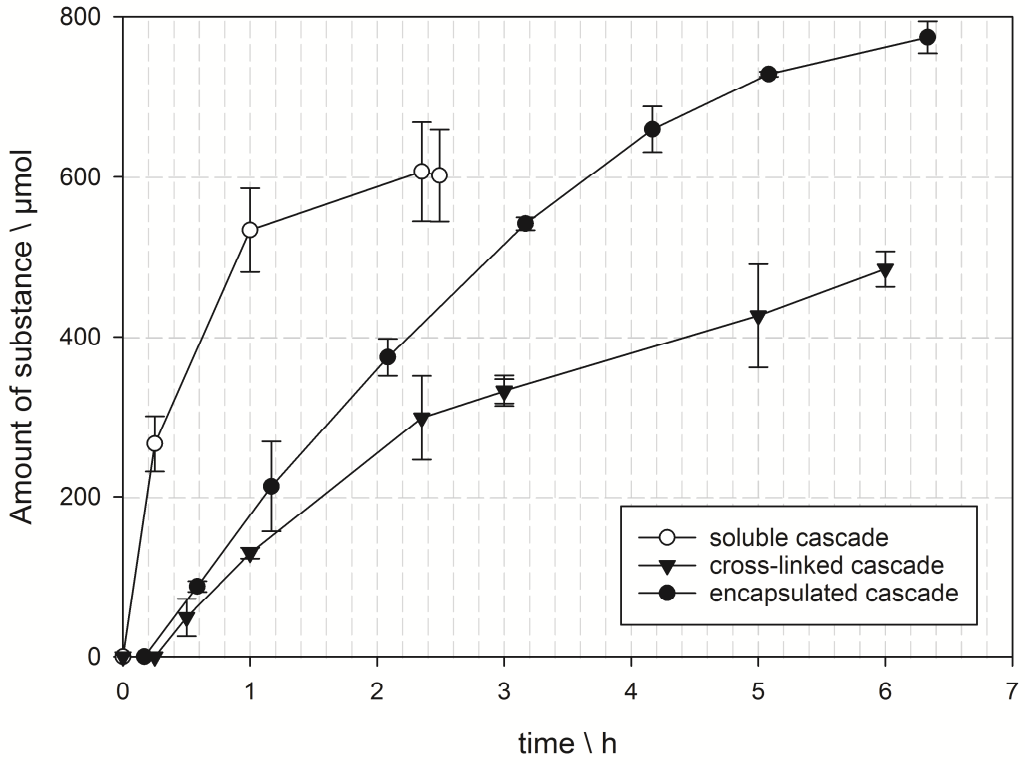
Fig. 2 Comparison of the soluble enzyme cascade (○) with two immobilization techniques, cross-linked enzyme aggregates (▼) and alginate encapsulation (●), used to establish a more stable reaction cascade. The results are displayed in µmol acetoin formed.

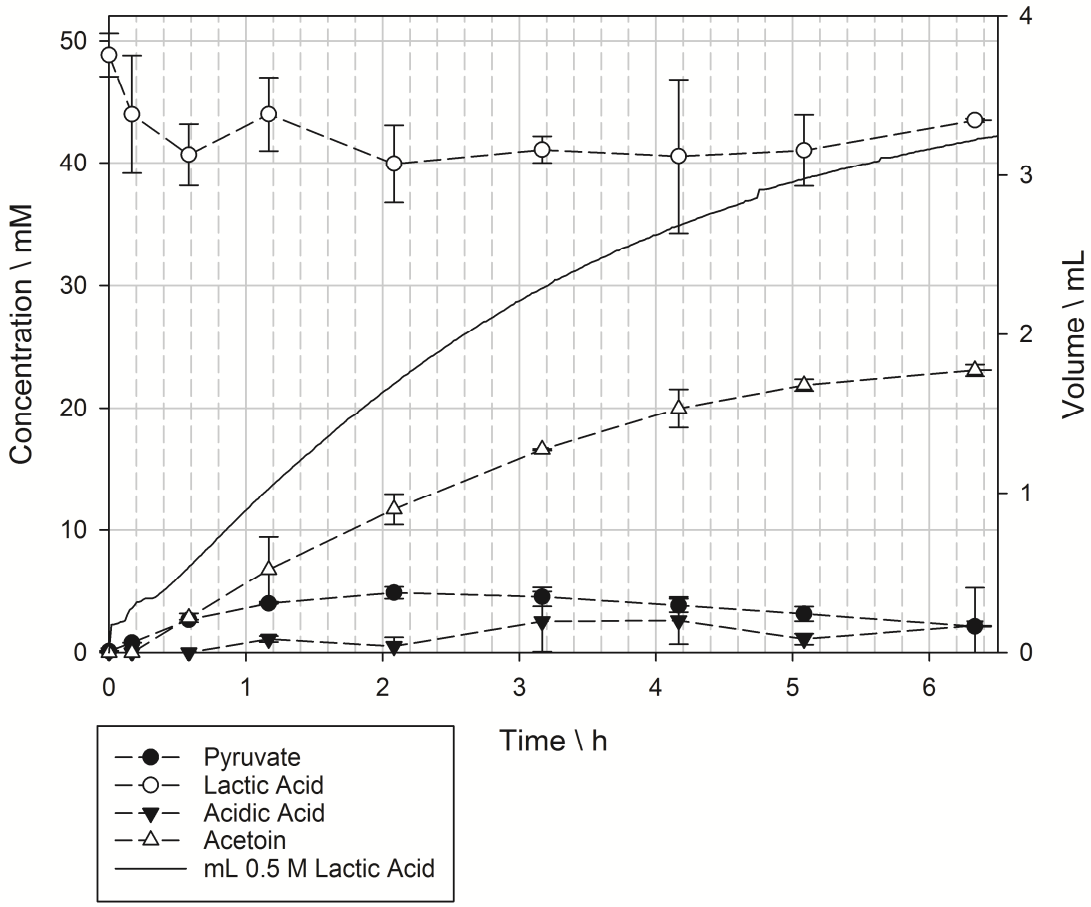
Fig. 3. Production of acetoin using an immobilized enzymatic reaction cascade, with lactic acid as titration reagent. All enzymes were entrapped in a 4% Ca-alginate polymer. The reaction was kept at 40 °C at a constant pH of 6.5 using 0.5 M lactic acid as titrant.

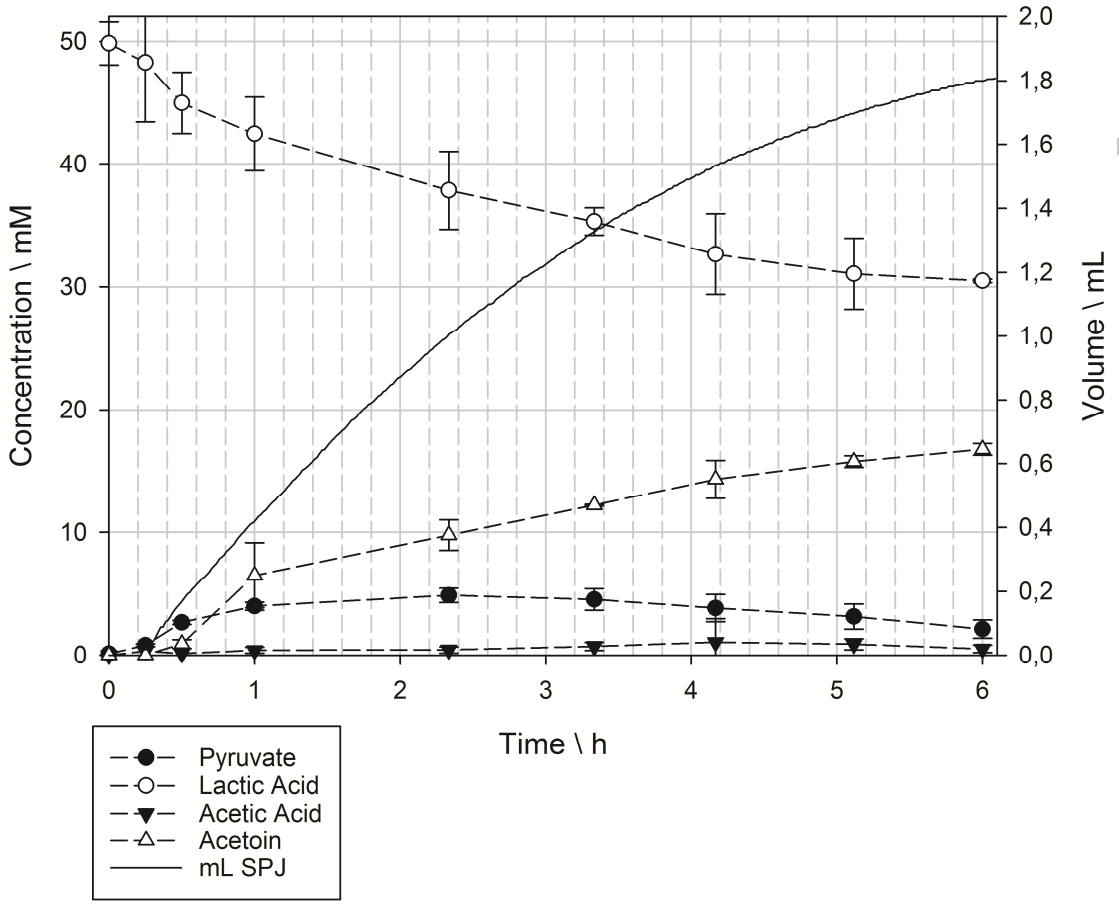
Fig. 4 Production of acetoin from lactic acid found in SPJ via an encapsulated enzymatic reaction cascade. Undiluted SPJ was used as the titration agent. The reaction was set up with 50 mM of pure lactic acid and run at 40 °C and a constant pH of 6.5.

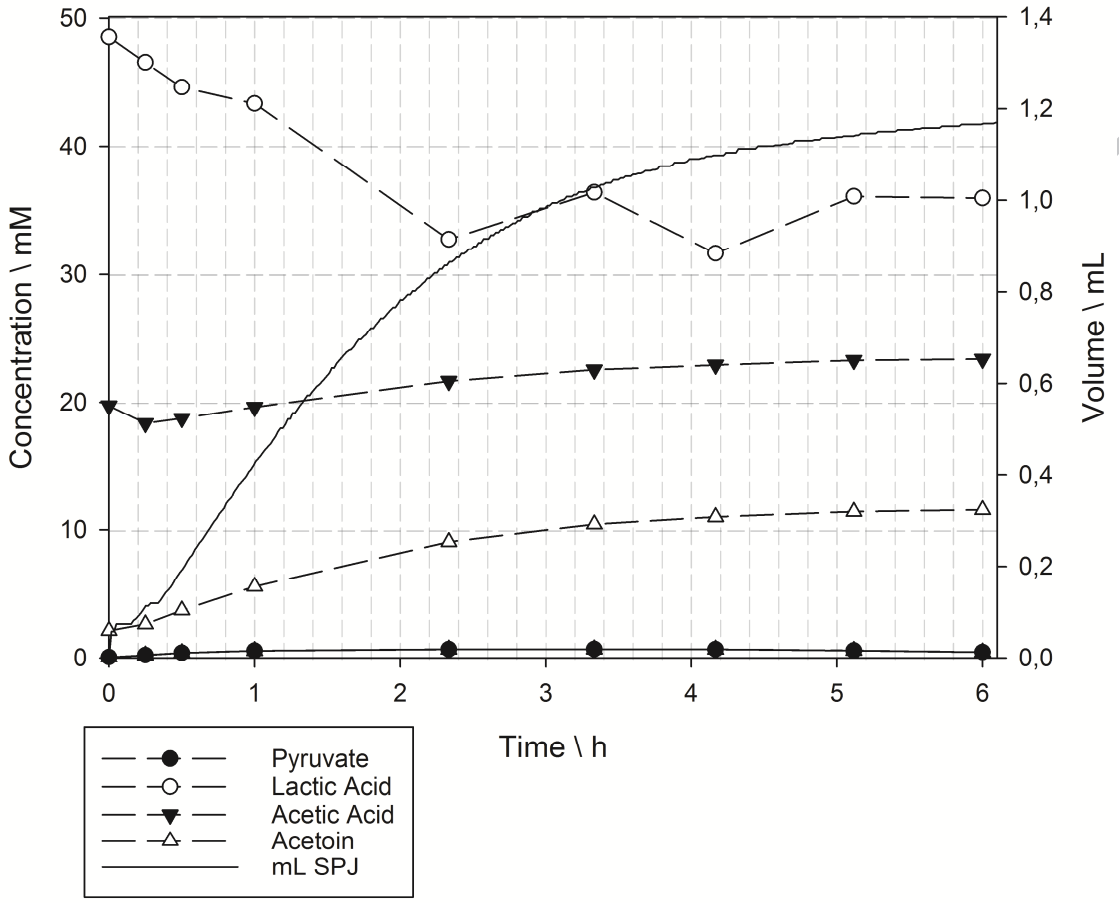
Fig. 5 Production of acetoin via encapsulated enzymatic reaction cascade using diluted SPJ (50 mM lactic acid) as initial mixture. The reaction was run at 40 °C and kept at pH 6.5 by titration of undiluted SPJ.











Highlights

Development of immobilized cell-free enzymatic cascade, systems biocatalysis

Utilization of lactic acid from pure undiluted grass silage press juice

Production acetoin and deacidification of grass silage press juice

Continuous pH controlled substrate supply in an oxygenated titration reactor vessel

Reaction duration of 6 h with 91% conversion of added lactic acid