Direct fermentation of potato starch and potato residues to lactic acid by *Geobacillus stearothermophilus* under non-sterile conditions

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Abstract

**BACKGROUND:** Lactic acid is an important biorefinery platform chemical. The use of thermophilic amylolytic microorganisms to produce lactic acid by fermentation constitutes an efficient strategy to reduce operating costs, including raw materials and sterilization costs.

**RESULTS:** A process for the thermophilic production of lactic acid by *Geobacillus stearothermophilus* directly from potato starch was characterized and optimized. *Geobacillus stearothermophilus* DSM 494 was selected out of 12 strains screened for amylolytic activity and the ability to form lactic acid as the major product of the anaerobic metabolism. In total more than 30 batches at 3–1 l scale were run at 60 °C under non-sterile conditions. The process developed produced 37 g L⁻¹ optically pure (98%) L-lactic acid in 20 h from 50 g L⁻¹ raw potato starch. As co-metabolites smaller amounts (<7% w/v) of acetate, formate and ethanol were formed. Yields of lactic acid increased from 66% to 81% when potato residues from food processing were used as a starchy substrate in place of raw potato starch.

**CONCLUSIONS:** Potato starch and residues were successfully converted to lactic acid by *G. stearothermophilus*. The process described in this study provides major benefits in industrial applications and for the valorization of starch-rich waste streams.

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**Keywords:** *Geobacillus stearothermophilus*; thermophiles; lactic acid; fermentation; starch; amylolytic bacteria

**INTRODUCTION**

Lactic acid (LA) has been widely used for decades in several industrial sectors such as food, pharmaceuticals, chemicals and cosmetics.¹ During the past 15 years LA gained additional importance as one of the top platform chemicals for the production of green solvents, fuel precursors and poly(lactic acid) (PLA), a biodegradable plastic.² As a result, by 2020 the global market of LA is expected to exceed 10⁹ kg.¹

Around 90% of the entire production of LA derives from fermentation, while chemical synthesis is no longer of significant importance.³ As claimed by Gao et al.,⁴ the two bottlenecks for the fermentative production of LA at industrial scale are the cost of the substrate (carbon source) and the operating costs, especially with regard to sterilization (sterilization equipment, energy consumption and labor cost). In order to reduce costs for the raw materials, starch represents among others (molasses, lignocelluloses, etc.) a cheap alternative to refined sugars.⁵ Starch from various origins can be used for LA production, including corn, cassava, potato and barley.⁶ Corn starch is a standard substrate commonly used for the production of LA at an industrial level.⁷ In these processes, however, the starch is not used directly but requires enzymatic hydrolysis to glucose prior to fermentation, since most of the LA bacteria cannot convert starch directly.⁸ Therefore, the utilization of amylolytic microorganisms which can grow directly on starch and convert it to LA represents an interesting option.⁹

The second issue raised by Gao et al. can be overcome by the application of thermophilic microorganisms like *Bacillus coagulans*, *Geobacillus stearothermophilus* (formerly known as *Bacillus stearothermophilus*) and *Bacillus licheniformis*⁵ which allows non-sterile fermentations operated at temperatures above 50 °C. The risk of contamination, which is of great concern especially at industrial scale, is reasonably low at such temperatures. It has of course to be argued that even if sterilization costs can be...
saved working at 60 °C, at the beginning of the process the fermentation medium has to be heated up. The desired temperature has also to be maintained during the lag phase. However, once growth starts most of the heating requirement is supplied by the metabolic heat released during the conversion of glucose to lactic acid. Other advantages of operating at high temperature (up to 60 °C) include: reduction in the energy input due to the cooling requirements during LA fermentation at lower temperatures and also the reduced solubility of oxygen at 60 °C in comparison with 37 °C, which facilitates the preservation of anaerobic conditions in the fermentation broth.

As can be expected, the combination of these desired qualities in one LA-producing strain is challenging. One good candidate is Geobacillus stearothermophilus, a facultatively anaerobic gram-positive bacterium of the phylum Firmicutes, with an optimal growth temperature between 50 and 60 °C and pH of 6.2–7.5. The species is well known as a producer of thermostable α-amylase and as a spore-forming microorganism used as a biological indicator for the deactivation of pathogens. Members of the species can ferment hexose and pentose sugars, glycerol and starch to produce lactate, acetate, formate and ethanol.

The thermophilic production of LA by G. stearothermophilus has previously been described by Danner et al., using sucrose as a substrate. Due to the well-known amylase activity of the species, we hypothesized that a similar process could be established even from raw starch. This would be also promising for biorefinery applications for the valorization of starch-rich waste streams, such as residues from agriculture and food processing, e.g. potato residues.

The primary objective of this study was the characterization and optimization of a non-sterile process for the thermophilic production of LA by G. stearothermophilus directly from raw potato starch and its application also to potato residues from food processing. This work provides new insights into the potential application of this species for LA fermentation also at an industrial level.

MATERIALS AND METHODS

Bacterial strains

Ten Geobacillus stearothermophilus strains were obtained from the Leibnitz Institute DSMZ – German Collection of Microorganisms and Cell Cultures. They are referred to by their DSM code number. In addition, two putative G. stearothermophilus strains were isolated from compost at the Institute for Environmental Biotechnology.

For conservation cultures were grown in shake flasks at 60 °C in a medium containing 8 g L⁻¹ nutrient broth (NB), 4 g L⁻¹ yeast extract (YE), 10 g L⁻¹ starch (or glucose) and Na₂HPO₄/KH₂PO₄ buffer (0.07 mol L⁻¹) at pH 7. Aliquots of 1 mL of these cultures in the late exponential growth phase (pH higher than 5.5) were prepared for long term storage at −80 °C with the addition of glycerol 10% v/v.

Media and carbon sources

Corn steep liquor (CSL), MRS broth (de Man, Rogosa, Sharpe) and nutrient broth were purchased from Sigma-Aldrich (Australia). Other medium components include: yeast extract type CMRT from Ohly GmbH (Germany) and soy peptone E110 (SP) from Organotechnie S.A.S. (France). Potato starch of technical grade and glucose were purchased from AGRANA (Austria). Potato residues were obtained from Trasa (Spain) and were collected from food-processing industries in the region of Navarra (Spain).

Potato residues: physical pretreatment and characterization

Potato residues consisted of a viscous mash of puree and peels. 100 g of fresh weight are composed of 86.7 ± 0.3 g moisture, 6.3 ± 0.1 g starch, 1.2 ± 0.0 g proteins and 0.5 ± 0.0 g total LA. 62 ± 1.1% of the LA in the residues was present as D-LA. In order to preserve the product from degradation the water content was reduced from 86.7 ± 1.4% to 13 ± 1.4% in an industrial drum dryer. The particle size was then reduced from c. 150 mm to c. 4 mm in diameter with a hammer mill. The total starch in the dry biomass as well as the amylose/amylopectin ratio were determined with enzymatic assay kits (K-TSTA 07/11 and K-AMYL 07/11, respectively, Megazyme, Ireland). The crude protein content was estimated from the total Kjeldahl nitrogen using a conversion factor of 6.25.

Fermentations

Preliminary tests were performed in 100 mL screw cap, non-baffled flasks (Schott Duran®, Schott, Germany) with a working volume of 50 mL. After sterilization these were inoculated with 0.5 mL of a cryoculture and incubated at 60 °C and 90 rpm in a rotary shaker (Infors HT Multitron, Switzerland). Culture conditions were not strictly anaerobic, but it is assumed that oxygen levels were close to zero during the growth phase. pH values were measured externally. Unless otherwise specified, the medium used was NB 8 g L⁻¹ and YE 4 g L⁻¹. All experiments were performed at least in duplicate.

Batch fermentations of raw potato starch were conducted in a Biostat ED 5-L fermenter (B. Braun Biotech International GmbH, Germany) with an initial working volume of c. 3 L. Two flat-bladed-disc-turbine impellers (six blades, outer diameter 70 mm) were mounted on the stirrer shaft and the agitation speed was set to 150 rpm. The initial starch concentration was c. 50 g L⁻¹. All experiments were performed at least in duplicate. Batch fermentations of potato residues were run in DASGIP parallel bioreactors of 1 L volume (DASGIP Information and Process Technology GmbH, Germany). The fermentation medium was prepared adding boiling water to the required amounts of SP, YE and dry potato residues for an initial working volume of 0.5 L. The experiments were performed in triplicate. The equivalent initial starch concentration was c. 56 g L⁻¹.

The fermentation medium (water, substrate and nutrients) was not aseptic (i.e. no sterilization applied) and the whole process was run under non-sterile conditions; this implies that feeding of the reactor and sampling took place from an upper port of the vessel by simply opening it to a non-sterile atmosphere. pH in the fermentation broth was regulated by controlled addition of NaOH 10 mol L⁻¹; the high concentration of the base was necessary to minimize dilution of the fermentation broth. Dissolved oxygen values in the liquid broth were monitored; however, no measures (such as sparging the broth with N₂) were taken to maintain

| Table 1. Definition of factors and levels in the experimental design for medium optimization in shake flask cultures. Levels are defined as low, medium and high. Concentrations are given in g L⁻¹ |
|---|---|---|
| Factors | Levels (g L⁻¹) |
| Yeast extract | low | medium | high |
| 2 | 4 | 6 |
| Soy peptone | 2 | 6 | 10 |
strict anaerobic conditions throughout the process. Nevertheless, dissolved oxygen levels remained close to zero from the early exponential phase due to the microbial activity. Yields of LA on starch were calculated as the difference between the final and the initial amount (g) of LA in the fermentation broth, divided by the total amount (g) of starch added at the beginning of the process.

**Multilevel factorial design for medium optimization**

In order to identify the optimal medium composition, a multilevel full factorial design (3^2) was applied. Nine complex media were defined based on two factors (YE, SP) and three levels (low, medium, high) as shown in Table 1. These were compared with the standard medium used for the cultivation of *G. stearothermophilus* based on NB and YE as already described. Significant differences between means were evaluated with a multiple range test according to Fisher’s least significant difference (LSD) at the 95% confidence level.

**HPLC analysis of sugars and organic acids**

Sugars and organic acids were determined via HPLC in a Hewlett Packard series 1100 system, equipped with an ion exclusion column ION 300 (Transgenomic®, USA) heated at 45 °C and a refractive index detector (Agilent 1100, Agilent technologies, USA). The mobile phase consisted of a 0.005molL^-1 H_2SO_4 solution with a flow rate of 0.325 mL min^-1.

**Other analytical methods**

In order to distinguish between the two optical isomers of LA an enzymatic assay kit (K-DLATE 06/08, Megazyme, Ireland) was used. Starch consumption was measured as the disappearance of blue color intensity given by the starch–iodine complex. One dextrinizing unit (U) is defined as the amount of enzyme that catalyzes the hydrolysis of 1mg of iodine binding starch per min in the assay conditions. The measured values are expressed as follows:

\[ U \text{ mL}^{-1} = \frac{\text{OD}_{620}^{\text{SAMPLE}} - \text{OD}_{620}^{\text{BLANK}}}{\text{mg STARCH} \times 20 \text{ min} \times 0.5 \text{ mL}} \]

The effect of pH on the amylase activity was investigated preparing starch solutions in different acetate or phosphate buffers (0.1molL^-1) to cover pH values in the range 5–8. All assays were performed in duplicate.

**RESULTS AND DISCUSSION**

**Strain selection**

A total of 12 strains were screened for amylolytic activity and for the ability to form LA as the major product of anaerobic metabolism. Results are presented in Table 2. For ten of these strains by the end of the batch test no residual starch was measured, which indicates high enzyme activity. The substrate was converted mainly to LA and to variable minor amounts of formic acid (FA), acetic acid (AA) and ethanol (EtOH). A few strains released higher fractions of by-products (e.g. IFA 102, DSM 485 and 304) and were therefore considered unsuitable for the purpose of this work. In the other cases, the concentration of LA reached within 48 h ranged from 3.42 to 4.26 g L^-1 from an initial amount of starch of 10 g L^-1 (Table 2). Despite complete liquefaction of the starch the observed yields were generally low. This can be attributed to the fact that the medium was not buffered and acid production resulted in a decrease of pH. Based on the total LA production and the relative amounts of by-products, the strain *G. stearothermophilus* DSM 494 was selected for further studies.

**Lactic acid batch fermentation from raw potato starch**

*Geobacillus stearothermophilus* DSM 494 was tested in 3–1 batch fermentations in a bioreactor at 60 °C in MRS medium. pH was incubated at 60 °C with 0.5 mL 1% w/v potato starch. After 20 min the reaction was stopped by the addition of 1 mL HCl 1 mol L^-1. Samples were transferred to 50 mL volumetric flasks and brought to volume with bi-distilled water and 2 mL of iodine (0.05% w/v I2, 0.5% w/v KI) to develop the blue color. The OD was read at 620 nm after 20 min. The method was adapted from the original version of Fuwa. One dextrinizing unit (U) is defined as the amount of enzyme that catalyzes the hydrolysis of 1 mg of iodine binding starch per min in the assay conditions. The measured values are expressed as follows:

\[ U \text{ mL}^{-1} = \frac{\text{OD}_{620}^{\text{SAMPLE}} - \text{OD}_{620}^{\text{BLANK}}}{\text{mg STARCH} \times 20 \text{ min} \times 0.5 \text{ mL}} \]

Results and discussion

| N  | Strain          | Residual starch | Lactic acid (g L^-1) | Formic acid (g L^-1) | Acetic acid (g L^-1) | Ethanol (g L^-1) | Final pH |
|----|----------------|-----------------|---------------------|----------------------|---------------------|-----------------|----------|
| 1  | DSM 297        | no              | 3.86 ± 0.07         | 0.39 ± 0.08          | 0.37 ± 0.10         | 0.17 ± 0.04      | 5.32 ± 0.04 |
| 2  | DSM 1550       | no              | 3.48 ± 0.02         | 0.17 ± 0.03          | 0.45 ± 0.01         | 0.11 ± 0.03      | 5.31 ± 0.00 |
| 3  | DSM 485        | yes             | 0.35 ± 0.04         | 0.00 ± 0.00          | 0.24 ± 0.02         | 0.00 ± 0.00      | 6.79 ± 0.01 |
| 4  | DSM 5934       | no              | 3.42 ± 0.38         | 0.17 ± 0.09          | 0.35 ± 0.02         | 0.07 ± 0.03      | 5.37 ± 0.04 |
| 5  | DSM 2313       | no              | 3.66 ± 0.10         | 0.12 ± 0.08          | 0.40 ± 0.01         | 0.13 ± 0.02      | 5.43 ± 0.02 |
| 6  | DSM 6790       | no              | 3.73 ± 0.20         | 0.20 ± 0.10          | 0.48 ± 0.12         | 0.11 ± 0.03      | 5.33 ± 0.01 |
| 7  | DSM 2357       | no              | 3.95 ± 0.05         | 0.30 ± 0.01          | 0.28 ± 0.02         | 0.16 ± 0.04      | 5.31 ± 0.06 |
| 8  | DSM 304        | yes             | 0.24 ± 0.00         | 0.00 ± 0.00          | 0.42 ± 0.01         | 0.00 ± 0.00      | 6.79 ± 0.01 |
| 9  | IFA 102        | no              | 3.02 ± 1.06         | 0.18 ± 0.35          | 0.86 ± 0.26         | 0.18 ± 0.05      | 5.86 ± 0.58 |
| 10 | IFA 9.1        | no              | 4.41 ± 0.04         | 0.27 ± 0.07          | 0.20 ± 0.01         | 0.26 ± 0.01      | 5.31 ± 0.01 |
| 11 | DSM 494        | no              | 4.26 ± 0.02         | 0.20 ± 0.01          | 0.25 ± 0.00         | 0.21 ± 0.00      | 5.37 ± 0.07 |
| 12 | DSM 22         | no              | 3.83 ± 0.03         | 0.32 ± 0.12          | 0.38 ± 0.03         | 0.09 ± 0.00      | 5.35 ± 0.00 |

Cultures grown in shake flasks on NB 8 g L^-1 - YE 4 g L^-1 medium with 10 g L^-1 raw potato starch. Errors are given as standard deviations (n = 2).
regulation and agitation enabled proper control of the conditions throughout the process, thus avoiding inhibition due to acidic conditions in the broth and prolonging the production of acid. In particular, it was possible to apply higher initial starch concentrations (50 g L\(^{-1}\)). With an inoculum of 1 mL volume the lag phase lasted c. 5–6 h.

During the exponential growth phase the starch was completely degraded, while the production of LA started until it stopped after approximately 24 h (Fig. 1). LA was produced up to a concentration of 31.9 g L\(^{-1}\). The measured enantiomeric purity of the L-LA by the end of the fermentation was 98%. Considering the impurities (D-LA present in complex substrate compounds, e.g. yeast extract) the optical purity of L-LA produced by the strain was 99.5%. The optical purity of the product exceeds the required standards for technical grade L-LA (usually >95% or >97%). However, it does not fulfill the strict specifications for polymer-grade LA, where a purity higher than 99% is required to guarantee good mechanical properties for PLA.\(^{21}\) It has to be addressed, however, that the reported values of optical purity of LA represent merely the ratio of the amount of L-LA over the total amount produced. These values are not indicative of the optical purity of the commercial product, since LA has not been purified from the fermentation broth.

The time course profile of LA production in this work using starch (Fig. 1) is comparable with the results reported by Danner et al.\(^ {18}\) using glucose as a substrate with the same strain. A detailed description of the levels of sugars (from the hydrolysis of starch) and of the metabolites produced during two parallel fermentations can be found in Table 3. It is evident that throughout the fermentation both glucose and maltose did not accumulate but were metabolized as soon as they were released. The minor products of the mixed acid fermentation (i.e. AA, EtOH and FA) were formed constantly during the fermentation and were not related to a specific growth phase. The maximum yield of LA was 47.9% ± 0.3%, which is partially due to the formation of co-metabolites. By the end of the fermentation process neither glucose nor starch was found in the broth, but residual maltodextrins could be observed in the chromatograms. This may be caused by a nutrient limitation or inhibitory effects exerted by the products. Therefore, a further medium optimization was conducted.

### Medium optimization
Yeast extract, corn steep liquor and soy peptone as sources of organic nitrogen and growth factors were chosen as suitable

| Time (h) | 0 | 3 | 6 | 8 | 10 | 12 | 14 | 25 |
|---------|---|---|---|---|----|----|----|----|
| Maltose (g L\(^{-1}\)) | 0.79 ± 0.01 | 0.79 ± 0.01 | 0.79 ± 0.01 | 0.70 ± 0.09 | 0.67 ± 0.03 | 0.41 ± 0.03 | 0.35 ± 0.03 | 0.29 ± 0.03 |
| Glucose (g L\(^{-1}\)) | 0.06 ± 0.02 | 0.06 ± 0.01 | < d.l. | < d.l. | 0.44 ± 0.03 | 0.08 ± 0.00 | 0.04 ± 0.02 | 0.03 ± 0.01 |
| Lactic acid (g L\(^{-1}\)) | 0.32 ± 0.01 | 0.31 ± 0.03 | 0.47 ± 0.00 | 1.75 ± 0.47 | 13.69 ± 2.46 | 23.78 ± 1.68 | 25.62 ± 0.18 | 31.91 ± 0.01 |
| Formic acid (g L\(^{-1}\)) | 0.76 ± 0.07 | 0.70 ± 0.02 | 0.80 ± 0.01 | 0.75 ± 0.04 | 1.76 ± 0.04 | 2.81 ± 0.02 | 2.77 ± 0.02 | 2.90 ± 0.04 |
| Acetic acid (g L\(^{-1}\)) | 3.70 ± 0.13 | 3.71 ± 0.04 | 3.61 ± 0.07 | 3.24 ± 0.41 | 3.73 ± 0.01 | 4.06 ± 0.06 | 3.91 ± 0.06 | 3.80 ± 0.04 |
| Ethanol (g L\(^{-1}\)) | 0.90 ± 0.02 | 0.89 ± 0.02 | 0.91 ± 0.01 | 0.92 ± 0.06 | 1.89 ± 0.08 | 2.94 ± 0.20 | 3.11 ± 0.01 | 3.71 ± 0.05 |

Concentrations (g L\(^{-1}\)) are corrected according to final volumes for the dilution effect of the NaOH 10 mol L\(^{-1}\) added. <d.l. ‘stands for’ below detection limits. Errors are given as standard deviations (n = 2).
candidates to fulfill the amino acid and vitamin requirements of *G. stearothermophilus*. Preliminary results obtained from shake flasks and from batch fermentations showed that CSL could not enhance the production of LA. In fact, CSL performed worse than MRS and NB (as positive controls). In combination with YE, CSL led to even lower yields (results not shown). Also the effect of further addition of MnSO₄ – described as essential to some key enzymes for the metabolism of *G. stearothermophilus* – was investigated, but appeared to be negligible (results not shown).

On the other hand, YE and SP showed a positive effect on LA production. The effects and the interactions of the two compounds were evaluated by a factorial experimental design.

The combination of SP and YE as nutrients for the production of LA provided highly satisfactory results. The statistical evaluation of the results obtained for cultures grown in shake flasks confirmed that there were significant differences in LA production among the 10 conditions tested (P-value of the F-test < 0.05). Moreover, as shown by the multiple range test at the 95% confidence level, almost all of the treatments performed better than the medium based on NB-YE used for conservation (Table 4). However, it appeared that too high concentrations of SP (10 g L⁻¹) had an inhibitory effect.

Some of the findings (e.g. the high yield of the SP 2 g L⁻¹-YE 2 g L⁻¹ test) appear to be inconsistent, which may be explained by the limitations of experiments in shake flasks. Therefore, the most interesting treatments (SP 2 g L⁻¹ - YE 2 g L⁻¹; SP 6 g L⁻¹ - YE 2 g L⁻¹; SP 6 g L⁻¹ - YE 6 g L⁻¹; SP 10 g L⁻¹ - YE 6 g L⁻¹) were tested in 3 L batch fermentations with pH control. The general trends observed in shake flasks were confirmed in this new set of experiments: with increasing amounts of SP and YE higher titers of LA and higher productivities were obtained (Fig. 2). In particular, on MRS medium as well as on NB-YE the final titer of LA was 33.0 g L⁻¹ (results not shown), similar to that obtained with SP 6 g L⁻¹ - YE 2 g L⁻¹. The production of LA rose to 36.6 g L⁻¹ with the medium SP 6 g L⁻¹ - YE 6 g L⁻¹, with an increase in yield up to 66.2% ± 2.0%.

As expected, increasing the dose of SP to 10 g L⁻¹ did not improve the titer and the yield, indicating that the vitamin and amino acid requirements were already fulfilled by the lower concentrated medium (SP 6 g L⁻¹ - YE 6 g L⁻¹). This is supported by the data illustrated in Table 5, which compares the amino acid content of the different media with the optimum composition, as described by Rowe *et al.* In extensive experiments the latter defined the amino acid composition of a medium that properly supported the aerobic growth of *G. stearothermophilus* NCA 1503. San Martin *et al.* focused on the requirements for growth of *G. stearothermophilus* LLD-15 under anaerobic conditions and concluded that methionine, isoleucine, serine and glutamate were

| SP (g L⁻¹) | YE (g L⁻¹) | LA (g L⁻¹) | Homogeneous groups |
|-----------|-----------|------------|-------------------|
| 2         | 2         | 11.41 ± 0.24 | def               |
| 2         | 4         | 9.57 ± 0.06  | bcd               |
| 2         | 6         | 9.72 ± 0.15  | cd                |
| 6         | 2         | 12.53 ± 0.82 | ef                |
| 6         | 4         | 10.77 ± 1.76 | de                |
| 6         | 6         | 13.12 ± 0.70 | f                 |
| 10        | 2         | 6.42 ± 0.15  | a                 |
| 10        | 4         | 7.28 ± 0.70  | a                 |
| 10        | 6         | 8.04 ± 0.47  | abc               |
| control   |           | 7.47 ± 0.02  | ab                |

Comparison with the medium used for preservation (control): NB 8 g L⁻¹ - YE 4 g L⁻¹. Errors are given as standard deviations (n = 2). Treatments with different letters in the homogeneous groups column have significantly different means (P > 0.05).

**Table 4.** Multiple range test (Fisher’s LSD, 95% confidence level) with soy peptone (SP) and yeast extract (YE) as factors affecting the production of lactic acid (LA) on raw potato starch.
Table 5. Free amino acid content in the four media based on soy peptone (SP) and yeast extract (YE) tested in batch fermentations

| Amino acid | ALA | ARG | ASN | ASP | CYS | GLU | GLN | GLY | HIS | ILE | LEU | LYS | MET | PHE | PRO | SER | THR | TRP | TYR | VAL |
|------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Reference (mg L⁻¹) | 84  | 64  | 50  | 130 | 400 | 50  | 50  | 50  | 42  | 100 | 164 | 140 | 52  | 86  | 100 | 140 | 84  | 30  | 56  | 126 |
| SP 2 - YE 2 (%) | 114 | 88  | -   | 35  | 8   | 56  | -   | 64  | 48  | 60  | 37  | 86  | 50  | 38  | 74  | 26  | 43  | 55  | 60  | 46  | 56  |
| SP 2 - YE 6 (%) | 162 | 169 | -   | 51  | 16  | 100 | -   | 88  | 86  | 92  | 106 | 60  | 126 | 34  | 74  | 83  | 100 | 82  | 87  |
| SP 6 - YE 6 (%) | 343 | 263 | -   | 106 | 24  | 168 | -   | 192 | 143 | 180 | 194 | 111 | 223 | 78  | 129 | 164 | 180 | 139 | 167 |
| SP 6 - YE 10 (%) | 390 | 344 | -   | 122 | 32  | 212 | -   | 216 | 181 | 212 | 235 | 134 | 274 | 86  | 160 | 193 | 220 | 175 | 198 | 126 | 400 | 50  | 50  | 42  | 100 | 164 | 140 | 52  | 86  | 100 | 140 | 84  | 30  | 56  | 126 |

Values according to the specifications in the technical data sheet of the respective products from Organotechnie. Reference as published by Rowe et al. 22 and values expressed as mg L⁻¹; all other values given as a percentage of the reference. SP6 - YE6 refers to a medium based on: SP 6 g L⁻¹ - YE 6 g L⁻¹.

Table 5 shows that SP 6 g L⁻¹ and YE 6 g L⁻¹ provided the minimal dose required, especially with regard to those amino acids characterized as essential.

A higher viscosity in the YE/SP medium after the addition of starch caused difficulties in the control of T and pH. Increasing the inoculum volume to 10% v/v resulted in a good initial amylolytic activity and a shortened lag phase (Fig. 2).

pH dependence of the alpha amylase activity

In agreement with the observations of Pfueller and Elliott 24 and Wind et al., 25 alpha amylase activity was found to be highest at pH 5.7 and reasonably good (above 90% of the maximum) in the range 5.3 – 6.7 (Fig. 3). At pH 7 the residual activity was 62.1%, but it decreased fast towards higher pH values. Activity values at a pH lower than 5 were considered irrelevant for these investigations, since all metabolic activities of DSM 494 are inhibited below this threshold value. For industrial production LA fermentations at lower pH levels are preferable because this reduces costs for the downstream processing. 6,26 Therefore, the observed optimum around pH 6 was considered very suitable. However, whether LA production was also optimal at pH 6 still had to be proven.

Influence of pH on LA fermentation

In a new set of experiments the impact of pH on LA production was investigated. As reported in Table 6, the production of LA (as well as co-metabolites) at pH 7 was higher than at pH 6. Final LA concentrations were 35% lower at pH 6 and 6.4 g L⁻¹ of unconverted glucose were found by the end of the fermentation. It is well known from the literature 12,27,28 that the toxicity of LA is more pronounced at lower pH levels due to the effect of the undissociated form of the acid. This is the obvious cause for the observed limitation of product formation at pH 6.

The molar ratios of the metabolites at pH 7 and 6 were considerably different, being 7.6 LA : 1.0 FA : 0.4 AA : 1.1 EtOH versus 18.3 LA : 1.0 FA : 0.0 AA : 3.4 EtOH, respectively. A similar behavior was described by Hartley and Shama 29 and San Martin et al., 30 who studied the anaerobic energy pathways of G. stearothermophilus, even though they focused on the fermentative production of EtOH. As suggested by these authors, the production and secretion of acids may be more difficult at lower pH since it goes against the pH gradient established by the cells between the extracellular and the intracellular environment to maintain the pH homeostasis. Moreover, acetic acid is a weaker acid than lactic acid and shows a stronger toxic effect at pH 6 due to the higher ratio of the undissociated form present already at high pH values.

At pH 8 the poor activity of the α-amylase resulted in high viscosity and the fermentation had to be aborted. For pH values above 7 the α-amylase activity of G. stearothermophilus drops quickly below 50% of the maximum (Fig. 3), therefore pH values higher than 7 should not be taken into consideration for this process.
Evaluation of process performances with raw potato starch as a C-source
The inhibitory effect of high concentrations of LA and of the co-metabolites was considered responsible for limiting the maximum LA concentration achievable at pH 7 to 37 g L\(^{-1}\). In fact, a similar value, around 40 g L\(^{-1}\), was also the maximum LA concentration reported by Danner \cite{7} with the same microorganism. As a result, the maximum yield of LA obtained in the optimized medium at pH 7 was 66.2\% and the total conversion of starch into LA and other metabolites was 77.8\% ± 2.6\%. Despite the undeniable limitation in yield, the process displays characteristics that are of particular interest concerning productivity and autoselectivity, as discussed below.

Among the examples of direct use of starch for LA production available in the literature and described in Table 7, Zhang and Cheryan \cite{31} reached a concentration of LA of 93 g L\(^{-1}\), which is the highest reported so far. However, the production strain Lactobacillus amylovorus yielded a mixture of the L- and D-LA. Son and Kwon \cite{32} also reported relatively high L-LA concentrations up to 71 g L\(^{-1}\) obtained from soluble starch by another Lactobacillus strain, namely L. manihotivorans, but in this case the productivity was very low: 0.6 g L\(^{-1}\) h\(^{-1}\). Fossi et al. \cite{33} used Lactobacillus fermentum achieving 53 g L\(^{-1}\) LA and a productivity of 1.1 g L\(^{-1}\) h\(^{-1}\); no claims were made concerning the optical purity of the product. A few examples about the use of fungi for the direct conversion of starch to LA are also listed in Table 7. Among them, the best results were obtained by Yuwa-amornpitak and Chookietwattana \cite{34} with Rhizopus microsporus reaching a concentration of 56 g L\(^{-1}\) pure L-LA and a productivity of 1.2 g L\(^{-1}\) h\(^{-1}\). In comparison, the process described in this study for the direct production of LA with G. stearothermophilus has a productivity of 1.8 g L\(^{-1}\) h\(^{-1}\), which is the second best only after Zhang and Cheryan \cite{31} and has the economic and technical advantage of requiring no sterilization and of being run under non-sterile conditions throughout the whole process. Within this study more than 30 runs at the given temperature of 60 °C and of 1–2 days duration were conducted and the process proved to be robust and not susceptible of contaminations. This was confirmed on the one hand by microscopic inspections, but more importantly by the fact that no change in the production pattern of the metabolites was observed between the replicates. This aspect is of great advantage at an industrial scale, where keeping aseptic conditions is difficult and linked to high costs, whereas non-sterile handling makes the process easier and flexible. Two examples of the production of LA in non-sterile conditions were recently reported by Ouyang et al. \cite{9} and Ma et al. \cite{35} The former achieved a concentration of 73.03 g L\(^{-1}\) of LA with a productivity of 1.04 g L\(^{-1}\) h\(^{-1}\) with a Bacillus strain from a lignocellulosic hydrolyzate. The latter produced 97 g L\(^{-1}\) L-LA (>99% optically pure) with Bacillus coagulans at 50 °C from an enzymatic hydrolysate of tapioca starch and excess sludge hydrolysate as a N-source; the reported productivity was 2.8–3.1 g L\(^{-1}\) h\(^{-1}\).

Even though a final concentration of 40 g L\(^{-1}\) of lactic acid is surely not attractive for industrial applications, the current process can be substantially improved by applying the following strategies. First, as already done in other studies focusing on ethanol production, \cite{10} it is possible to knock out the genes responsible for the production of the co-metabolites. In this way, lactic acid yields can be increased. Moreover, the volumetric productivity of the process may be increased in continuous fermentations with membrane bioreactors. The fact that the process does not require strict sterile conditions in order to run stable would be of further advantage in this context. Finally, by integrating the purification step...

### Table 6. Product formation and residual glucose (g L\(^{-1}\)) by the end of batch fermentations in the optimized medium with raw potato starch or potato residues as a C-source

| pH | Lactic acid (g L\(^{-1}\)) | Formic acid (g L\(^{-1}\)) | Acetic acid (g L\(^{-1}\)) | Ethanol (g L\(^{-1}\)) | Residual glucose (g L\(^{-1}\)) |
|----|----------------|----------------|----------------|----------------|----------------|
| 6  | Raw potato starch | 23.84 ± 0.54 | 0.67 ± 0.01 | 0.00 ± 0.08 | 2.27 ± 0.03 | 6.43 ± 0.05 |
| 7  | Raw potato starch | 36.62 ± 0.05 | 2.47 ± 0.30 | 1.17 ± 0.68 | 2.72 ± 0.07 | 0.47 ± 0.13 |
| 7  | Potato residues  | 59.64 ± 0.28 | 0.52 ± 0.00 | 3.76 ± 0.48 | 1.92 ± 0.01 | 0.99 ± 0.34 |

Errors are given as standard deviations (n = 2 or n = 3).

### Table 7. Examples of direct conversion of starch to lactic acid (LA) available in the literature

| Microorganism | LA (g L\(^{-1}\)) | Yield (%) | Productivity (g L\(^{-1}\) h\(^{-1}\)) | L-LA purity | T (°C) | Sterile | Reference |
|---------------|----------------|-----------|-------------------------------|-----------|-------|--------|----------|
| Lactococcus lactis* | 19 | 94 | 0.8 | high (−%) | 30 | yes | Bhanwar et al. \cite{37} |
| Lactobacillus fermentum | 53 | - | 1.1 | - | 45 | yes | Fossi et al. \cite{33} |
| Lactobacillus paracasei | 37 | 93 | 0.8 | 92.5% | 45 | yes | Petrova and Petrov \cite{38} |
| Enterococcus faecium | 17 | 93 | 1.1 | 98.6% | 30 | yes | Shibata et al. \cite{39} |
| Lactobacillus manihotivorans | 71 | 85 | 0.6 | 100.0% | 30 | yes | Son and Kwon \cite{42} |
| Lactobacillus amylophilus | 33 | 85 | 0.3 | high (−%) | 37 | yes | Vishnu et al. \cite{40} |
| Rhizopus oryzae | 36 | 87 | 0.4 | 100.0% | 28 | yes | Xiao et al. \cite{41} |
| Rhizopus microsporus | 56 | - | 1.2 | 100.0% | 40 | yes | Yuwa-amornpitak and Chookietwattana \cite{34} |
| Lactobacillus amylovorus | 93 | 77 | 2.4 | low (−%) | 40 | yes | Zhang and Cheryan \cite{31} |
| Geobacillus stearothermophilus | 37 | 66 | 1.8 | 98.0% | 60 | no | This study |

*in a dialysis bioreactor.
with the fermentative process (e.g. by ion exchange, electrodialysis or extraction) it would also be possible to reduce the amount of NaOH necessary to neutralize the product formed, since this would be continuously removed from the fermentation broth.\(^{36}\)

The present work is the first example of LA fermentation boasting the combined features of the direct conversion of starch and non-sterile operational conditions.

**Fermentation of potato residues as a C-source**

When raw starch in the optimized medium was substituted with untreated potato residues as a C-source, *G. stearothermophilus* succeeded in the hydrolysis of the starchy substrate and its conversion to LA, as shown in Fig. 4 and Table 6. The given value for the starch concentration at the beginning of the process is lower than the actual total starch concentration in the medium because only the dissolved fraction could be measured. Beside 6.3% starch the potato residues also contained 0.5% LA. Therefore, the initial LA concentration in the fermentation medium was 11.9 g L\(^{-1}\) ± 0.35 g L\(^{-1}\) (46.5% L-LA). Consequently, although L-LA was produced during the course of the fermentation with an optical purity of 99.5%, the overall amount of L-LA by the end of the process was 89.5%.

The time-course profile of the first 20 h of fermentation resembled the observations at similar conditions (\(T = 60^\circ\)C, pH 7) on raw potato starch, with the only difference being the initial LA concentration. As evident in Fig. 4, after 18 h the production of LA stopped once an LA concentration of 40 g L\(^{-1}\) was achieved. This behavior was observed in previous experiments (data not shown) independently from the carbon source and also at higher substrate concentrations. In contrast to the experiments with raw starch, in this case a lag phase of 5 h occurred, during which glucose accumulated. After this period the production of LA restarted with a similar pattern of metabolites, until a concentration of 59 g L\(^{-1}\) LA was reached after 48 h. The process was run for another 12 h, with little increase in the production: 60 g L\(^{-1}\) LA was reached after 60 h. The total productivity during the first 18 h was 1.6 g L\(^{-1}\) h\(^{-1}\) and it decreased to 1.0 g L\(^{-1}\) h\(^{-1}\) considering a production period of 48 h.

Apparently the lag-phase served as a period of adaptation for the strain to high concentrations of LA, once the threshold value of 40 g L\(^{-1}\) was reached. This behavior was never observed with raw potato starch as a substrate even after 48 h but may depend on the presence of additional nutrients provided by the potato residues. A higher fraction of the substrate may have been more easily available to the strain due to structural differences in the starch. Measurements showed that the percentage of amylase in the potato residues was 47% compared with 26% in the potato starch. Therefore, the starch in the potato residues is degraded to a higher extent and more fermentable sugar is released by the bacterial amylases. This explains the release of additional glucose during the lag phase after 18 h and the higher overall LA concentration in this case. Consequently the yield of LA at 48 h was 81.2%, which is considerably higher than the results obtained for raw starch (66.2%).

**CONCLUSIONS**

An effective process for the direct conversion of starch to lactic acid with *G. stearothermophilus* DSM 494 at 60°C was developed. 37 g L\(^{-1}\) optically pure (98%) L-lactic acid were obtained in 20 h from 50 g L\(^{-1}\) raw potato starch. As co-metabolites smaller amounts of acetic acid, formic acid and ethanol were formed. In comparison with most literature data on similar direct fermentation processes high productivity (1.8 g L\(^{-1}\) h\(^{-1}\)) was obtained from raw potato starch. Moreover, the high growth temperature of the selected species provided distinct techno-economic advantages, e.g. no sterilization of the medium required, non-sterile operational conditions and a lower cooling demand throughout the

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**Figure 4.** Time-course profile of starch consumption and lactic acid production during batch fermentations of potato residues as a C-source in soy peptone-yeast extract medium at 60°C and pH 7. Error bars indicate the standard deviation (\(n = 3\)).
fermentation. Finally the suitability of the process was demonstrated using an actual ‘zero value’ waste product, potato residues from food processing. With this substrate even higher lactic acid concentrations (59 g L\(^{-1}\)) were achieved with a yield of 81.2%.

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