CHEMICAL ENGINEERING TRANSACTIONS
VOL. 110, 2024
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Marco Bravi, Antonio Marzocchella, Giuseppe Caputo
Copyright © 2024, AIDIC Servizi S.r.l.
ISBN 979-12-81206-10-6; **ISSN** 2283-9216
DOI: 10.3303/CET24110053

Influence of Different External Liquids on the Process Outcomes of Lactic Fermentation in Suspended Capsules

Giulia Lentini[a,+,*], Francesca Passannanti[a,+], Marianna Gallo[b], Rosa Colucci Cante[a], Federica Nigro[c], Roberto Nigro[a], Andrea Luigi Budelli[d]

a Department of Chemical Engineering, Materials, and Industrial Production, University of Naples Federico II, P. Tecchio 80, Naples-Italy.
b Department of Industrial Engineering, University of Niccolò Cusano, Via Don Carlo Gnocchi 3, Rome-Italy.
c I. T. P. Innovation and Technology Provider S.r.l., Via Bisignano a Chiaia, 68 80121 Naples-Italy.
d Heinz Innovation Center, Nieuwe Dukenburgseweg 19 6534 AD Nijmegen Postbus 57 NL-6500, The Netherlands
+ Contributed equally to the work.
giulia.lentini@unina.it

Encapsulation is an innovative promising technique in the field of biotechnology, both for confining live microorganisms to promote their viability during processing and for the target delivery in the gastrointestinal tract, and for trapping active metabolites produced during a fermentation process within a carrier material, allowing their gradual release. During this work, a probiotic strain, Lacticaseibacillus paracasei CBA L74, was encapsulated using a hydrolyzed oatmeal suspension as a prebiotic carrier, by extrusion technique with $CaCl_2$ a (1M) and alginate (1% w/v). The mini-bioreactors obtained were suspended in three different external liquids, (the same hydrolyzed oatmeal suspension used for the capsule production; a hydrolyzed oatmeal suspension with a modified pH equal to 3.3, and a hydrolyzed oatmeal suspension formulated by adding a specific concentration of dead bacteria) to evaluate whether the external liquid can help the confinement of the bacteria in the capsules. For this purpose, the fermentation process was carried out at 37°C for 24h; both capsules and external liquids were characterized by microbiological and chemical analyses. The maximum bacterial growth (10^9 CFU/mL) was reached in all the capsules, except for those suspended in the formulation with a modified pH where a bacterial charge of 3.5×10^8 CFU/mL was obtained; in any case, the bacterial confinement was not obtained. The highest lactic acid concentration (15.9 g/L) was observed for the capsules suspended in the same oatmeal suspension used for encapsulation, while the lowest one was obtained in the external oatmeal liquid with a modified pH.

1. Introduction

In bioprocesses, such as fermentation, one of the most important objectives is to maximize the efficiency and yield of the process. Microbial immobilization through encapsulation is a new technique for building up a higher cell density in the bioreactor. Cell retention techniques, that include cell recycling (Brandberg et al. 2007), cross-linking of cells (Abelyan 2000), entrapment in a matrix (Taherzadeh et al., 2001), and encapsulation in a polymer gel membrane (Talebnia and Taherzadeh, 2006), offer some advantages, such as the easy product recovery by separation of the cells from the metabolites produced during the fermentation process, the increase in the volumetric productivity and also the reduction of the overall cost (Westman et al., 2012). The most widely used encapsulating material is alginate, a linear heteropolysaccharide of d-mannuronic and l-guluronic acid extracted from various species of algae (Smidsrod, Haug, & Lian, 1972). Alginate capsules can be produced by extrusion technique, obtaining a gel matrix with a pore size of less than 17 nm (Klein et al., 1983) good for containing bacteria during the fermentation process (Krasaekoopt et al., 2004). Alginate-based capsules are carriers capable of releasing or confining bacteria and bioactive compounds in a controlled manner. Furthermore, the rate of the compound release can be controlled, acting on different parameters, such as varying the concentration of cross-linking agent during gel formation (Gerbsch and Buchholz, 1995) or reducing pushing forces of solutes and cells (Liu et al., 2008). In the present work, hydrolyzed oatmeal suspension was chosen

Paper Received: 01 March 2024; Revised: 21 March 2024; Accepted: 19 May 2024
Please cite this article as: Lentini G., Passannanti F., Gallo M., Colucci Cante R., Nigro F., Nigro R., Budelli A., 2024, Influence of different external liquids on the process outcomes of lactic fermentation in suspended capsules , Chemical Engineering Transactions, 110, 313-318 DOI:10.3303/CET24110053

as fermentation substrate for its prebiotic nature to produce capsules by direct extrusion (1% alginate and 1M calcium chloride) to entrap a probiotic strain, Lacticaseibacillus paracasei CBA L74. The capsules were suspended in three different external liquids (the same hydrolyzed oatmeal suspension used for the capsule production; a hydrolyzed oatmeal suspension with a modified pH (3.3), and a hydrolyzed oatmeal suspension formulated by adding a specific concentration of dead bacteria) to study both the influence of different external liquids on the process outcomes of a lactic fermentation using minibioreactors and the confinement of bacteria in the capsules. During the fermentation process, which was carried out for 24 hours, both the capsules and the external liquid were characterized in terms of bacterial growth and lactic acid production. To deeply characterize their surface morphology, a confocal microscopy analysis of the capsules (1% alginate and 1M calcium chloride) was carried out.

2. Materials And Methods

2.1 Strain and feedstock

Lacticaseibacillus paracasei CBA L74 was used as a probiotic strain for fermentation trials. It was patented and provided by Heinz Italia S.p.A. It was stored at -80 °C in cryovials with glycerol (20%) and reactivated through incubation at 37°C for 24 h in 9 mL of an animal-free broth (20 g/L Bacto Yeast Extract, BD Biosciences; 0.5 g/L MgSO4, Sigma-Aldrich; 50 g/L Glucose, Sigma-Aldrich; 0.5 g/L citric acid, Sigma-Aldrich) before inoculation. The cell density in the inoculum broth was 10^8 CFU/mL. Whole oatmeal (Le Farine Magiche, Lo Conte Group) was purchased in a local store in Naples, Italy.

2.2 Substrates preparation

A hydrolyzed oatmeal suspension was prepared to produce the minibioreactors and the three different external liquids in which capsules were suspended during the fermentation process. The oatmeal suspension (15% w/v of oat flour with 1% w/v of added glucose) was pre-treated by enzymatic hydrolysis using amylase (0.054% w/v) to avoid starch gelling during the sterilization process, without affecting the mixing in the fermentation process as reported by Gallo et al. (2021). The sterilization phase was conducted in an autoclave 134°C for 40 min as mentioned by Lentini et al. (2022).

2.3 Encapsulation process

The capsules were produced by direct extrusion with the modified protocol according to Sáez-Orviz et al. (2021). The hydrolyzed oatmeal suspension was mixed with Na-alginate (0.01 g/mL) and the strain had an initial bacterial concentration of about 5 x 10^6 [CFU/mL]. The mixture was dropped, through a peristaltic pump into a calcium chloride solution (1M) for 20 minutes at room temperature. The nozzle sizes and the flow rate were 2 mm and 5 mL/ min, respectively. Then capsules were collected and rinsed in distilled water for a few minutes.

2.4 Fermentation protocol

Fermentation tests were carried out in falcon with a total volume of 20 mL. A fixed volume of 5 mL of the capsules was suspended in 15 mL of the external liquid. To study the process, the capsules were suspended in three different external liquids:

1. Same hydrolyzed oatmeal suspension to avoid pushing forces of the solutes origin;
2. Same hydrolyzed oatmeal suspension with a modified pH equal to 3.3 to avoid pushing forces of the solute origin and to get stronger chain interactions of the alginate's microstructure (Bennacef et al., 2021);
3. Same hydrolyzed oatmeal suspension with the addition of dead bacteria in a concentration equal to that of live bacteria inoculated into the capsules, to avoid pushing forces of solutes and bacterial origin.

The fermentation process was carried out using a wheel as an agitation system and incubating the falcon at 37°C, without pH control. In Table 1, a schematic description of all the samples investigated is summarized.

Table 1. Description of the investigated samples

Fermentation Process	Samples	Description
1	C_1	Hydrolyzed oatmeal CAPSULES
	L_1	Hydrolyzed oatmeal EXTERNAL LIQUID
2	C_2	Hydrolyzed oatmeal CAPSULES
	L_2	Hydrolyzed oatmeal EXTERNAL LIQUID with a modified pH of 3.3
3	C_3	Hydrolyzed oatmeal CAPSULES
	L_3	Hydrolyzed oatmeal EXTERNAL LIQUID by adding dead bacteria

Analytical methods

Bacterial count, lactic acid, and secondary metabolite determination

Before analyses, capsules were broken in 1% (w/w) sodium citrate solution at pH 6.

The bacterial concentration found in both capsules and external liquid has been monitored, sampling at time 0 and after 2, 4, 6, 16, 18, 20, 22, and 24 h from the beginning of the fermentation. Night sampling was impossible, causing a lack of data between time 6 and time 16. Samples were then serially diluted and spread-plated on Petri plates filled with De Man, Rogosa, and Sharpe (MRS) agar (Oxoid, Basingstoke, UK). To control the presence of microbial contaminants in the samples, MacConkey agar (Oxoid, Basingstoke, UK) and Gelatin Peptone Bios Agar (Biolife, Milan, Italy) were used. All plates were incubated at 37 °C for 24 h before reading. Anaerobic kits (Anaerogen Compact, Oxoid, Basingstoke, UK) were used for MRS plates to guarantee anaerobic growth conditions for Lacticaseibacillus paracasei CBAL74 during the incubation period. The bacterial count was expressed in CFU/mL, where CFU is the colony-forming unit.

The concentration of lactic acid of both capsules and external liquid samples was determined at the end of the process (T24) by high-performance liquid chromatography (HPLC), Agilent Technologies 1100, equipped with an Agilent Synergi Hydro-RP C18 column (250 mm × 4.6 mm and a pore size of 4 µm) with a visible/UV detector. The mobile phase consisted of 0.27% (w/v) KH2PO4 aqueous solution at a pH=1.5 modified with H3PO4 with a column temperature of 60 °C and a flow rate of 1 mL/min. The detection was set at 210 nm.

Secondary metabolites, such as acetic, propionic, and butyric acids, were measured by gas chromatography, (GC), Agilent technologies 6890, using a capillary Poraplot Q column (25 mm × 0.32 mm). The mobile phase was helium gas with a flow rate of 200 mL/min.

Morphology characterization of the capsules

Capsules were cut and morphologically analyzed on both the external and internal surfaces.

The morphology characterization of the capsules was performed using a confocal laser microscope (confocal laser microscope Leica TCS SP8 X on a Leica DMI8 microscope; Leica Microsystems, Germany) equipped with a camera lens of 63x/1.40 oil. For the analyses, the autofluorescence of the samples has been exploited. A white laser (λ excitation of 490 nm and λ emission of 581–687 nm) was employed. Leica Application Suite X version (1.8.1 Copyright 1997-2015 Leica Microsystems CMS GmbH) as acquisition software was used to analyze the images. The capsules before the analysis were cut into two parts to study the internal and external structure.

2.5 Statistical Analysis

Statistical analysis was performed using Prism 9®. Fermentation tests and analyses of lactic acid and secondary metabolite were carried out in triplicate and, for each experimental data, mean values, and standard deviations (n = 3) were calculated. Their statistical significance was evaluated by one-way ANOVA followed by Tukey's multiple comparisons test, accepting as significant only results with $p < 0.05$.

3. Results And Discussion

3.1 Fermentation results

The fermentation process was carried out under controlled conditions of temperature (37 ◦C), suspending 5 mL of capsules in 15 mL of external liquid for 24 hours in agitation on a wheel. The bacterial growth curve for C_1, L_1, C_2, L_2, C_3, and L_3 samples is shown in Figure 1.

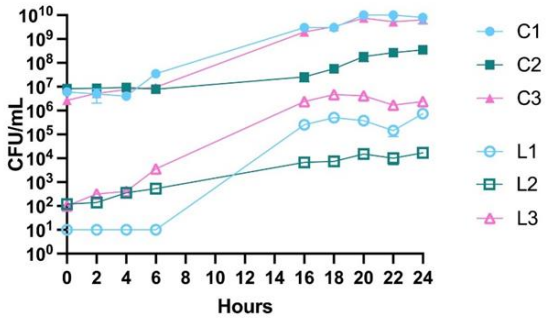

Figure 1: Bacterial growth curves of C_1, L_1, C_2, L_2, C_3, and L_3 samples determined during 24 hours of fermentation. Each result reported is a mean value of a triplicate analysis and error bars indicate the corresponding standard deviations.

The bacterial growth curves (Figure 1) showed the efficiency of the C_1, C_2, and C_3 minibioreactors as fermenters. C_1 and C_3 showed the same growth trend, starting the stationary phase after 16 hours of fermentation and maintaining a constant bacterial load of 9 log until the end of the process (24h). In the case of the C_2 microbioreactors the stationary phase begins after 20 hours of fermentation, reaching the maximum bacterial growth of $3.5 \times 10^8 \pm 5.5 \times 10^7$ CFU/ mL, an order of magnitude lower than the C_1 and C_3 samples. Although the difference was not statistically significant, it was probably due to the acidity of L_2 in which C_2 was suspended, which inhibits the bacterial growth in the capsules. Although the capsules acted as minibioreactors in all the external liquids in which they were suspended, none of them managed to confine the bacteria inside the capsules during the process. In the modified external liquids L_2 and L_3, the bacterial concentration was not negligible ($1.7 \times 10^2 \pm 2.5 \times 10$ CFU/ mL and $1.0 \times 10^2 \pm 2.6 \times 10$ CFU/ mL, respectively), at time zero. In all three external liquids (L_1, L_2, and L_3) at the end of the process a non-negligible bacterial concentration equal to $7.4 \times 10^5 \pm 4.3 \times 10^4$ CFU/ mL, $1.7 \times 10^4 \pm 4.3 \times 10^3$ CFU/ mL and $2.4 \times 10^6 \pm 3.6 \times 10^5$ CFU/ mL was detected respectively. This means that the spread of the bacteria was not reduced despite the external liquids being varied to minimize the pushing forces of bacterial and solute origin and by modifying the pH to stabilize the structure of the capsules. Moreover, no significant quantities of acetic, propionic, and butyric acids were detected (data not shown), indicating the absence of contaminants during the process.
Lactic acid concentration for C_1, L_1, C_2, L_2, C_3, and L_3 samples after 24 hours of fermentation is shown in Figure 2.

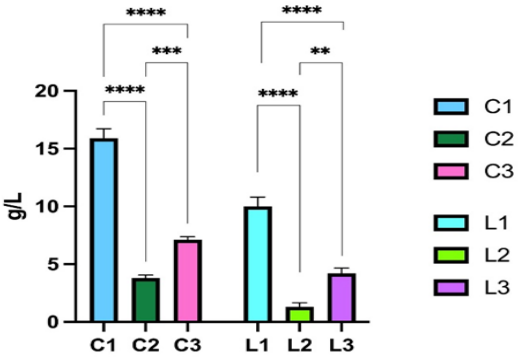

*Figure 2: Lactic acid concentration of C_1, L_1, C_2, L_2, C_3, and L_3 samples determined after 24 hours of fermentation. Each result reported is a mean value of a triplicate analysis and error bars indicate the corresponding standard deviations. Values marked with **, ***, and **** are significantly different ($p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively).*

In all capsules C_1, C_2, and C_3 and external liquids L_1, L_2, and L_3 the lactic acid content was present after 24 hours of fermentation. It was evident that as the external liquids vary, the lactic acid content both in the capsules and in the external liquids changes in a statistically significant way. In formulation 1 in which the hydrolyzed oatmeal capsules (C_1) were suspended in the same oatmeal suspension used for the capsules (L_1) was reached the maximum lactic acid content, equal to 15.90 ± 0.81 g/L and 10.00 ± 0.80 g/L, respectively. Bahry, Hajar, et al. (2019) valorized a solid carob waste from the Lebanese industry by optimizing the production of lactic acid

using Lactobacillus rhamnosus immobilized in alginate pearls obtaining after the fermentation process a value of 22 g/L inside the beads, slightly higher but comparable to that produced in C1 capsules studied in this work. Therefore, formulations 2 and 3 in which the external liquids have been modified, affected in a negative way the production of lactic acid as the metabolism of the lactobacillus was inhibited.
To the best of our knowledge, no one has studied the liquid system in which mini-fermentation reactors were suspended to understand the influence of different external liquids both on the process outcomes of lactic acid fermentation and on the confinement of bacteria in the capsules. In this regard, according to Bennacef et al. (2021), the pH of the external liquid of formulation 2 was modified to 3.3 to get stronger chain interactions of the alginate's microstructure of the capsules to try to confine the bacteria. The external liquid of formulation 3 was also modified adding dead bacteria to avoid concentration gradients and favoring the confinement. Although it was tried to minimize the pushing forces of solutes and bacterial origin, the release of the bacteria was not blocked. According to Petraitytė, Sigita, and Aušra Šipailienė (2019) after 24 hours of fermentation with Lactobacillus plantarum F_1 in alginate and calcium chloride capsules a comparable bacterial charge of 9 log CFU/mL, was reached. As previously stated by Dembczynski, R., and T. Jankowski (2000), the concentrations of Lactobacillus acidophilus cells, encapsulated in alginate/starch liquid-core capsules increased their population after 30 hours of fermentation from 8.3 x 10^7 to 6.5 x10^{10} CFU/mL, reaching a same delta growth as that of the capsules studied in this work.

3.2 Morphology characterization of the capsules

To understand the role of the oatmeal structure on the capsules' morphology autofluorescence, a spontaneous emission due to endogenous fluorophores present in vegetal cells, was exploited.

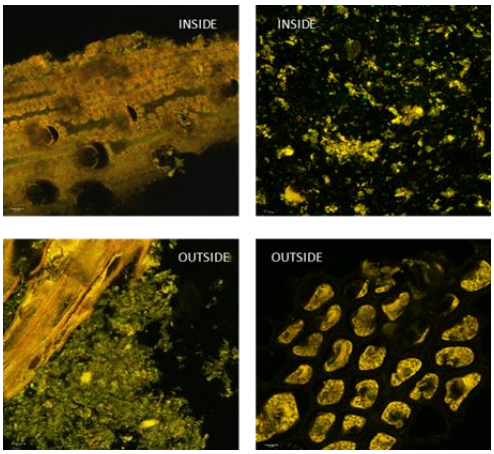

Figure 3: Internal and external structure of hydrolyzed oatmeal capsules

Confocal microscopic images (Figure 3) reveal amounts of oat-based structures such as starch granules, fiber fragments, and unidentified residues, as also reported by Wood et al. (2002) which studied the autofluorescence of compounds present in fermented oat and wheat bran. Therefore the interaction of the hydrolyzed oatmeal suspension with the alginate which was not possible to view as it does not exhibit autofluorescence, allows to create an overall coating of the capsules characterized by a non-homogeneous and compact structure.

4. Conclusions

The aim of this work was the production of probiotic hydrolyzed oatmeal capsules by direct extrusion and their use as mini fermenters. Three different external liquids, in which capsules were suspended to carry out the fermentation process, were studied to understand their role in the fermentation metabolism and bacterial confinement. Already from time zero, there was a release of bacteria, albeit minimal, which increased in the following 24 hours of fermentation. The external liquids tested were therefore not able to promote the confinement of the bacteria into the capsules. However, the capsules work well as minibioreactors reaching a final bacterial load of around 9 logs at the end of the fermentation process. Even if it was not possible to confine the bacteria with formulation 1, the process was made more efficient both in capsules C_1 and in the external liquid L_1, reaching a concentration of lactic acid equal to 15.90 ± 0.81 g/L and 10.00 ± 0.80 g/L, respectively. Capsules were further characterized by confocal microscopy, allowing us to appreciate the integration between the alginate and the oatmeal in the creation of the coating. Prospects will be to try to confine bacteria inside the

minibioreactors testing formulations in which the capsules have double coatings, thus forming tighter and more compact meshes that help in the confinement. Moreover, a scale-up of the minibioreactors process must be designed to evaluate the fermentation and its possible application in the industrial field.

References

Abelyan, V. A., 2000, A new method for immobilization of microbial cells by cross-linking, Applied Biochemistry and Microbiology, 36, 310-314.

Bahry, H., Abdalla, R., Pons, A., Taha, S., & Vial, C., 2019, Optimization of lactic acid production using immobilized Lactobacillus Rhamnosus and carob pod waste from the Lebanese food industry, Journal of biotechnology, 306, 81-88.

Bennacef, C., Desobry-Banon, S., Probst, L., & Desobry, S., 2021, Advances on alginate use for spherification to encapsulate biomolecules, Food Hydrocolloids, 118, 106782.

Brandberg, T., Gustafsson, L., & Franzén, C. J. ,2007, The impact of severe nitrogen limitation and microaerobic conditions on extended continuous cultivations of Saccharomyces cerevisiae with cell recirculation, Enzyme and Microbial Technology, 40(4), 585-593.

Dembczynski, R., & Jankowski, T. (Ed), 2000, Growth of lactic acid bacteria in alginate/starch capsules, In Progress in Biotechnology, Vol. 17, Elsevier 291-294.

Gallo, M., Passannanti, F., Schioppa, C., Montella, S., Colucci Cante, R., Nigro, F., Budelli, A.L. & Nigro, R., 2021, Enzymatic pretreatment and lactic fermentation of wheat flour suspension at a high solid content, Journal of Food Processing and Preservation, 45(4), e15299.

Gerbsch, N., & Buchholz, R., 1995, New processes and actual trends in biotechnology, FEMS Microbiology Reviews, 16(2-3), 259-269.

Klein, J., Stock, J., & Vorlop, K. D.,1983, Pore size and properties of spherical Ca-alginate biocatalysts, European journal of applied microbiology and biotechnology, 18, 86-91.

Krasaekoopt, W., Bhandari, B., & Deeth, H., 2004, The influence of coating materials on some properties of alginate beads and survivability of microencapsulated probiotic bacteria, International dairy journal, 14(8), 737-743.

Lentini, G., Nigro, F., Colucci Cante, R., Passannanti, F., Gallo, M., Budelli, A. L., & Nigro, R., 2022, Functional Properties of an Oat-Based Postbiotic Aimed at a Potential Cosmetic Formulation, Fermentation, 8(11), 632.

Liu, C. H., Wu, J. Y., & Chang, J. S., 2008, Diffusion characteristics and controlled release of bacterial fertilizers from modified calcium alginate capsules, Bioresource technology, 99(6), 1904-1910.

Petraitytė, S., & Šipailienė, A., 2019, Enhancing encapsulation efficiency of alginate capsules containing lactic acid bacteria by using different divalent cross-linkers sources, Lwt, 110, 307-315.

Sáez-Orviz, S., Passannanti, F., Gallo, M., Colucci Cante, R., Nigro, F., Budelli, A. L., Rendueles, M., Nigro, R. & Díaz, M., 2021, Lactic acid bacteria co-encapsulated with lactobionic acid: probiotic viability during in vitro digestion, Applied Sciences, 11(23), 11404.

Smidsrød, O., Haug, A., Lian, B., Huhtikangas, A., Pearson, W. B., & Meisalo, V.,1972, Properties of Poly (1, 4-hexuronates) in the Gel State. II. Comparison of Gels of Different Chemical Composition, Acta Chemica Scandinavica, 26, 79-88.

Taherzadeh, M. J., Millati, R., & Niklasson, C., 2001, Continuous cultivation of dilute-acid hydrolysates to ethanol by immobilized Saccharomyces cerevisiae, Applied biochemistry and biotechnology, 95, 45-57.

Talebnia, F., & Taherzadeh, M. J., 2006, In situ detoxification and continuous cultivation of dilute-acid hydrolyzate to ethanol by encapsulated S. cerevisiae, Journal of biotechnology, 125(3), 377-384.

Westman, J. O., Ylitervo, P., Franzén, C. J., & Taherzadeh, M. J., 2012, Effects of encapsulation of microorganisms on product formation during microbial fermentations, Applied microbiology and botechnology, 96, 1441-1454.

Wood, P. J., Arrigoni, E., Miller, S. S., & Amadò, R., 2002, Fermentability of oat and wheat fractions enriched in β-glucan using human fecal inoculation, Cereal chemistry, 79(3), 445-454.