Biotechnology Progress

GALACTO-OLIGOSACCHARIDES AND LACTULOSE AS PROTECTANTS AGAINST DESICCATION OF LACTOBACILLUS DELBRUECKII SUBSP. BULCARICUS

Journal:	*Biotechnology Progress*
Manuscript ID:	BTPR-14-0068.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	01-Jul-2014
Complete List of Authors:	Santos, Mauricio; Center for Research and Development in Food Cryotechnology (CIDCA CCT-La Plata), Araujo-Andrade, Cuauhtémoc; Universidad Autónoma de Zacatecas, Unidad Académica de Física Esparza-Ibarra, Edgar; Universidad Autónoma de Zacatecas, Unidad Académica de Biologia experimental Tymczyszyn, E; Center for Research and Development in Food Cryotechnology (CIDCA CCT-La Plata), Gomez-Zavaglia, Andrea; Center for Research and Development in Food Cryotechnology (CCT-CONICET La Plata),
Keywords:	Lactobacillus delbrueckii subsp. bulgaricus, Desiccation, Galacto-oligosacharides, Near infrared spectroscopy, Damage

GALACTO-OLIGOSACCHARIDES AND LACTULOSE AS PROTECTANTS AGAINST DESICCATION OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULCARICUS*

Mauricio I. Santos[a], Cuauhtémoc Araujo-Andrade[b], Edgar Esparza-Ibarra[c], Elizabeth Tymczyszyn[a] and Andrea Gómez-Zavaglia[a1]

[a] *Center for Research and Development in Food Cryotechnology (CCT-CONICET La Plata), RA-1900, Argentina*

[b] *Unidad Académica de Física. Universidad Autónoma de Zacatecas. Zacatecas, MX 98000 Mexico.*

[c] *Unidad Académica de Biologia experimental. Universidad Autónoma de Zacatecas. Zacatecas, MX 98000 Mexico*

[1]Corresponding author. Tel.: +54 221 4254853; Fax: +54 221 4249287
E-mail address: angoza@qui.uc.pt

Abstract

Lactobacillus delbrueckii subsp. *bulgaricus* CIDCA 333 was dehydrated on desiccators containing silica gel in the presence of 20% w/w of two types of galacto-oligosaccharides (GOS Biotempo and GOS Cup Oligo H-70®) and lactulose, until no changes in water desorption were detected. After rehydration, bacterial growth was monitored at 37 ºC by determining: a) the absorbance at 600 nm; b) the near infrared spectra (NIR). Principal Component Analysis (PCA) was then performed on the NIR spectra of samples dehydrated in all conditions. A multiparametric flow cytometry assay was carried out using carboxyfluorescein diacetate and propidium iodide probes to determine the relative composition of damaged, viable and dead bacteria throughout the growth kinetics.

The absorbance at 600 nm and the position of the second derivative band at ~1370 nm were plotted against the time of incubation. The efficiency of the protectants was GOS Biotempo > GOS Cup Oligo H-70® > lactulose. The better protectant capacity of GOS Biotempo was explained on the basis of the lower contribution of damaged cells immediately after rehydration (t=0). PCA showed three groups along PC1, corresponding to the *lag*, exponential and stationary phases of growth, which explained 99% of the total variance. Along PC2, two groups were observed, corresponding to damaged or viable cells.

The results obtained support the use of NIR to monitor the recovery of desiccated microorganisms in real time and without the need of chemical reagents. The use of GOS and lactulose as protectants in dehydration/rehydration processes was also supported.

Keywords: *Lactobacillus delbrueckii* subsp. *bulgaricus*; Desiccation; Galacto-oligosachharides; Lactulose; Near infrared spectroscopy; Multiparametric flow cytometry; Damage; Viable cells.

INTRODUCTION

The importance of lactic acid bacteria in the elaboration of dairy products underlines the necessity of an adequate preservation. The decrease of water activity is responsible for cell damage during preservation and lipid membranes are the first targets of dehydration injury. This loss of water is responsible for both bacteria damage and loss of viability, both of them finally delaying the fermentation processes [1,2]. Therefore, the use of protective compounds is crucial to avoid damage during dehydration [3-8]. In the last years certain sugars, like galacto-oligosaccharides (GOS) and lactulose have demonstrated a good protective properties [7,8].

Two hypotheses were proposed to explain the protectant capacity of sugars: vitrification and capacity to replace water molecules [9,10]. The vitrification hypothesis explains protection mediated by sugars on the basis of their capacity to form glassy states [sugars with high vitreous transition temperatures (Tg) are better protectants because they contribute to maintain cells in a vitreous state at storage temperatures] [9,10]. The hypothesis of water replacement considers that sugars directly interact with the polar heads of lipids in the dried state decreasing the membrane phase transition temperature (Tm). Hence, membranes dehydrated in the presence of sugars remain in the liquid crystalline phase as if they were hydrated [9,10]. These hypotheses are not excluding. On the contrary, it has been reported that in several cases that vitrification is a necessary but not sufficient condition for a good protection. This explains why polysaccharides with high Tg (*i.e.*: maltodextrines, starch) are not good protectants. Moreover, some articles suggest that the conjoint use of high Tg polysaccharides with small sugars having not so high Tg but that interact with membranes (*i.e.:* glucose or sucrose) may be a good protection strategy [4,9].

The recovery of desiccated bacteria is an important issue when evaluating the protective capacity of sugars. Therefore, monitoring growth kinetics after rehydrating the dehydrated microorganisms should ideally provide complete information about the evolution of bacteria recovery. *Lag*, exponential (*log*) and stationary phases of growth are characterized by different biochemical reactions leading to the synthesis of the cellular components required for bacteria growth and division [11]. For example, the *log* phase is defined as the balanced growth stage because the average composition of the cells (*i.e.*, protein and DNA) remains constant with bacterial culture properties

increasing at the same rate. When the stationary phase is reached, there is no longer a net increase in viable bacterial cell numbers and cellular metabolic activity is decreased meaning that the growth rate is equal to that of the death rate. During the transition between *log* and stationary phase, cellular components are synthesized at unequal rates. For this reason, the biochemical composition of cells in the stationary phase is different from that in the *log* phase [11]. All these biochemical changes can be followed in real time using spectroscopic methods [11,12].

Methods based on vibrational spectroscopy, including near infrared spectroscopy (NIR) are particularly useful because of their feasibility for monitoring biological processes without the necessity of exogenous chemical reagents, which saves costs and time. In addition, the ease to interface spectroscopic equipments to almost any computer converts NIR in a useful technique for automatic control, quality assessment or process optimization in real time [13,14]. Spectral features in NIR provide a rich source of information arising from combinations and overtones of the fundamental vibrations associated with C-H, O-H and N-H bonds. However, the interpretation of the huge amount of information contained in the spectra requires the use of chemometric methods. This enables a dynamic monitoring of industrial processes *in situ*, in environments consisting of complex chemical mixtures [13,15-17]. In this regard, NIR has been used to detect and monitor microorganisms in food-related applications [18-31].

Multiparametric flow cytometry (MFC) is a powerful technique that allows discrimination of microorganisms in different physiological states. The combined use of carboxyfluorescein diacetate (cFDA) and propidium iodide (PI) probes enables discrimination of viable, dead and damaged cells [32]. cFDA passively diffuses into cells and intracellular esterases cleave its acetate groups to yield highly green fluorescent carboxyfluorescein succinimidyl ester. The charged red fluorescent dye PI labels DNA, but only penetrates bacterial cells with damaged membranes [32].

In this work, the role of lactulose and two types of GOS as protectants against dehydration was evaluated. *Lactobacillus delbrueckii* subsp *bulgaricus* CIDCA 333 was dehydrated in desiccators containing silica gel until no changes in water desorption were detected. Bacterial growth after rehydration was followed using NIR and absorbance at 600 nm. Results were interpreted using an integrated analysis including: a) MFC and b) principal component analysis of the NIR spectra.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus delbrueckii subsp. *bulgaricus* CIDCA 333 was isolated from a fermented product [33]. The strain was stored frozen in 120 g/L non-fat milk solids at -80 ºC. Cultures were grown at 37 ºC in MRS broth [34].

GOS

Two types of GOS containing mixtures of galactose and glucose as monomers were studied: GOS Cup Oligo H-70® (Kowa Company, Tokyo, Japan) kindly donated by Kochi S.A. (Santiago, Chile) and GOS Biotempo, kindly donated by Biocircle Road S.L. (Porto, Portugal). GOS Cup Oligo H-70® contained GOS of different degree of polymerization (DP) whose relative composition was: high-molecular-weight oligosaccharides (DP≥5), 4%; tetrasaccharides (DP 4), 21%; trisaccharides (DP 3), 47%; disaccharides (DP2) and lactose, 23%, and monosacharides, including glucose and galactose, 5% [7]. The relative composition of GOS Biotempo was: pentasaccharides (DP5), 8%; tetrasaccharides (DP4), 42% and trisaccharides (DP3), 47% [35].

Lactulose (4-O-β-D-Galactopyranosyl-(1→4)-β-D-fructofuranose)

Commercial lactulose, disaccharide resulting from isomerization of lactose, was utilized (Discovery fine chemicals, Dorset, UK).

Drying over silica gel procedure

One mL cultures in the stationary phase (grown overnight at 37 °C in MRS broth, to attain *ca.* 10^9 CFU/ mL) were harvested by centrifugation (10 min at 4000 *g*). The pellets were washed twice with 20% (w/w) solutions of GOS Cup Oligo H-70®, GOS Biotempo and lactulose, previously sterilized using 0.2 µm sterile filters, or with 0.85% w/v sodium chloride (control).

The pellets were kept on the centrifuge tubes and dried over desiccators containing silica gel, until no changes in water desorption were detected as measured gravimetrically (approximately 7 days).

Growth kinetics

Desiccated microorganisms were rehydrated for 15 min at 25 °C in 1 mL of 0.85% (w/v) sodium chloride. The rehydrated microorganisms were inoculated in 10 mL of MRS broth and incubated at 37 ºC. Growth kinetics were followed by determining:

a) The absorbance at 600 nm every 60 min for each condition assayed.

b) The NIR spectra every 60 min. The spectra were collected *in situ* in the 900-1700 nm spectral region. An Ocean Optics spectrophotometer (model NIRQuest 512; Dunedin, Florida USA) equipped with a thermoelectrically cooled charge-coupled device (CCD) in a linear array of 512 pixels (optical resolution: 2 nm; full width at half maximum (FWHM) and signal:noise ratio equal to 15000:1) (Dunedin, Florida USA) was used. NIR absorption spectra were registered under a transmission configuration, using two optic fibers of low OH content (400 µm of core diameter). One of them was used to transmit the light towards the sample and other one (addressed to the detector), to collect the transmitted light. The liquid sample was placed in a quartz cuvette located in a holder connected to the optical fibers. The integration time for collecting NIR spectra was set at 5 msec and for each time of incubation, five spectra were registered. As radiation source, a tungsten-Halogen lamp (model LS-1, Ocean Optics Company) was used. The power output of the lamp (vis/NIR range) was 6.5 watts. SpectraSuite™ software (Ocean Optics, Dunedin, Florida USA) was used to register the experimental spectra.

Data Analysis

PCA was performed over the raw NIR spectra using The Unscrambler® software (version 9.8, CAMO, Norway). The spectra corresponding to the growth kinetics of bacteria desiccated in all

conditions (with GOS Biotempo, GOS Cup Oligo H-70®, lactulose and without protectants) and those of the controls were used for the analysis.

Flow cytometry analyses

MFC was used to determine viable, damaged and dead microorganisms throughout each kinetic of growth. cFDA and PI probes (Molecular Probes. Invitrogen SA, CA. USA) were used according to Raoult *et al.* (2007) [32]. For each assay, 1 mL of culture was harvested, washed twice and resuspended in 1 mL milli-Q water. The suspensions were incubated with 1 µl cFDA (50 µg/µL) at 28 °C in the dark for 10 min. Afterwards PI was added to a final concentration of 0.5 mg/mL and incubated at room temperature for 5 min.

Determinations were carried out with a FACS Calibur instrument using the CellQuest software (Becton Dickinson, Mountain View, CA, USA) according to Raoult *et al.* (2007) [32]. For each sample 10,000 events were collected, being the event rate less than 300 events/s. All parameters were collected as logarithmic signals. FL1 channel (530 nm) was used to set the green fluorescence of cFDA and FL3 channel (670), to set the red fluorescence of PI. Mixtures of thermally dead cells (80°C for 30 min) and freshly harvested cells were stained with cFDA and PI both in double-staining assays. They were used as controls to set the flow cytometer detectors and compensation, to differentiate four regions: Q1 (dead bacteria): PI+ and cFDA-; Q2 (membrane damaged bacteria): PI+ and cFDA+; Q3 (debris): PI- and cFDA-; and Q4 (viable bacteria): PI- and cFDA+. The percentage of each population was determined as [i/(Q1 + Q2 + Q4)] /100, where i is Q1, Q2, or Q4. No fluorescent debris (Q3) was excluded [36].

Reproducibility of results

Three sets of experiments performed using three different cultures of microorganisms were carried out. In each set, samples were prepared in duplicate. The relative differences were reproducible independently of the set of cultures used. Results obtained for duplicates were also reproducible.

RESULTS

The plate counts of dehydrated *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333 immediately after rehydration (time of incubation equal to zero) were: 2.00×10^5 CFU/mL, 6.30×10^4 CFU/mL and 2.00×10^4 CFU/mL for cells desiccated with GOS Biotempo, GOS Cup Oligo H-70® and lactulose, respectively. Microorganisms desiccated without protective compounds dropped to less than 10^4 CFU/mL after rehydration.

Figure 1 shows the kinetics of growth of microorganisms desiccated in the presence of GOS Biotempo, GOS Cup Oligo H-70® and lactulose, followed by absorbance at 600 nm. Microorganisms dehydrated in the absence of protectants were not able to grow after 30 h of incubation. The efficiency of the protectants was GOS Biotempo > GOS Cup Oligo H-70® > lactulose, as determined by the increase of the *lag* time (15, 20 and 21 h, respectively).

The NIR spectra registered throughout all the kinetics of growth are depicted in Figure 2 (raw spectra in Scripts I and inverted second derivative spectra in Scripts II). The 1300-1400 nm region was the most sensitive to the evolution of growth kinetics. In particular, the inverted second derivative band at ~1370 nm depicted a continuous shift to higher wavenumbers for the control (Figure 2AIII). On the contrary, no changes were observed for microorganisms dehydrated without protectants, indicating a correlation between spectral overlap and no growth of microorganisms (Figure 2BIII). For microorganisms dehydrated with sugars, spectra registered at the beginning of the kinetics fully overlapped. At intermediate times of incubation, the shift of the ~1370 nm band was gradual, as that observed in the control. At the end of the kinetics spectral changes were slighter (Figure 2 A,C,D,E scripts III).

Figure 3 shows more clearly the shift of the ~1370 nm band along with the growth kinetics, fitting nicely the kinetics of growth depicted in Figure 1 in the *lag*, *log* and early stationary phases. In the late stationary phase, a slight shift of the analyzed band was observed. This shift was only observed for the controls, which were the only cells that attained the late stationary phase after 30 h of incubation (see circled squares in Figure 3).

The evolution of viable, dead and damaged cells throughout the kinetics of growth followed a similar pattern in all the conditions assayed (Figure 4). The relative contribution of viable, damaged and dead cells in the *lag*, *log* and early stationary phase of growth was mainly determined by the contribution of viable and damaged cells, as that of dead cells was around 10% (Figures 4A-D). The contribution of dead cells was higher for microorganisms desiccated without protectants (Figure 4E).

Immediately after rehydration (time equal to zero) cells were mostly damaged. The repair of that damage, occurring during the *lag* phase, leaded to a continuous increase of viable cells, which attained maximum values at the beginning of the *log* phase (70-80% of viable cells in the *log* phase in all conditions) (Figures 4). These values remained unchanged until the early stationary phase. In the late stationary phase, damaged and dead cells (that up to that moment remained around 10%) started increasing at expenses of viable cells. This observation was particularly clear in the controls (Figure 4A), the only cultures that attained the late stationary phase after 30 h of incubation. To obtain a full picture of the evolution of viable, damaged and dead cells, MFC determinations were followed until 46 h of incubation. This allowed us to obtain information about viable, damaged and dead cells in the late stationary phase also for desiccated microorganisms.

The differential protectant capacity of GOS and lactulose could also be observed from the MFC assays. Indeed, the percentage of damaged cells immediately after rehydration (time equal to zero) was 44, 65 and 76% for cells desiccated with GOS Biotempo, GOS Cup Oligo H-70® and lactulose, respectively (Figures 4B,C,D).

PCA was carried out over all the NIR spectra registered throughout the five kinetics of growth (control, cells dehydrated with GOS Biotempo, GOS Cup Oligo H-70® and lactulose, and without protectants). Figure 5 depicts the PC2 *vs* PC1 scores plot, where PC1 explains 99% of the total variance. Three groups were observed along PC1-axis (Figure 5). One of them, containing a high amount of samples in a quite restricted space along PC1-axis, corresponded to microorganisms in the *lag* phase (opened circles (***a***) in Figure 5). The second group was the most heterogeneous along PC1-axis and corresponded to bacteria in *log* phase (opened triangles (***b***) in Figure 5). The third group was homogeneous along PC1-axis and contained samples in the stationary phase (opened squares (***c***) in

Figure 5). The dash line in the plot separates two groups along PC2-axis, corresponding to situations where bacteria were mostly damaged (***d***) or mostly viable (***e***). We will come back to this issue in the Discussion section.

DISCUSSION

The use of prebiotics like GOS and lactulose as protective compounds is very recent [7,8,37,38]. Therefore, for the interpretation of their protectant capacity, several physical chemical aspects need to be analyzed.

When investigating the efficiency of a given compound as protectant against desiccation, the recovery of cells after rehydration is an important issue that cannot be underestimated. For this reason, growth kinetics carried out on desiccated microorganisms are useful to analyze the evolution of this recovery. In this regard, NIR spectroscopy provided rich information about bacterial recovery after dehydration-rehydration (Figures 2,3,5). PCA allowed not only the discrimination of microorganisms in different phases of growth, but also the discrimination related with bacterial damage. The high percentage of variance explained in PC1-axis (99%) indicated the accuracy of NIR to discriminate microorganisms in different phases of growth (Figure 5). In particular, the high density of the cluster corresponding to microorganisms in the *lag* phase indicated the extremely low variability of bacteria spectra in this phase of growth (Figures 2 and 5). The high overlapping of spectra corresponding to microorganisms dehydrated without protectants (that did not leave the *lag* phase after 30 h of incubation) is very graphic in this respect (Figure 2BI). Microorganisms in the stationary phase of growth also grouped in a dense cluster along PC1-axis, correlating the moderately low variability of spectra in this phase (Figure 5 group ***c***). On the contrary, the higher variability of spectra corresponding to microorganisms in the exponential phase gave a heterogeneous group along PC1-axis (Figure 5 group ***b***).

On the other hand, two groups were observed along PC2-axis, that could be ascribed to damaged (or dead) and viable cells (Figure 5, groups ***d*** and ***e***). The analysis of MFC supported this interpretation (Figure 4). In the *log* phase microorganisms were mostly viable (70-80%). This explains

that the heterogeneous group along PC1-axis was very homogeneous along PC2-axis (Figure 5). On the other hand, damaged cells predominated in the *lag* phase (Figure 4). This explains that cells in this phase of growth belonged to the damaged group along PC2-axis (Figure 5, groups ***b*** and ***e***). Regarding microorganisms in the stationary phase, part of them grouped with the damaged cells and part of them with the viable ones. The co-existence of viable and damaged cells (mostly viable in the early stationary phase, with an increase of damaged cells in the late stationary phase) explains this observation (Figures 4 and 5 ***c,d,e***). It is important to point out that the slight shift of the second derivative band at ~1370 nm in the late stationary phase of growth (circled squares in Figure 3), where damaged and dead cells start increasing (Figure 4A) was a further indication of the physiological information provided by NIR spectroscopy. All these observations reinforce the usefulness of NIR to easily obtain physiological information in real time without the need of exogenous reagents.

The information provided by MFC assays also explained the different protectant capacity of both GOS and lactulose. If considered that once attained the *log* phase, bacteria are mostly viable (about 70-80% in all cases, including controls) and that it is during the *lag* phase when microorganisms repair their damage (damaged cells decreased during this phase of growth in all conditions) (Figure 4), one can logically conclude that the length of the *lag* phase was determined by the decrease of damaged cells during the *lag* phase. This decrease is conditioned by the percentage of damaged cells immediately after rehydration (time equal to zero). Hence, the lower the percentage of damaged cells after rehydration, the lower the time required to repair them. In this regard, after rehydrating microorganisms desiccated with GOS Biotempo, GOS Cup Oligo H-70® and lactulose, the percentage of damage was 44, 65 and 76%, respectively (Figures 4B,C,D). This supports the greater protectant capacity of GOS Biotempo (shorter *lag* time) (Figure 1, 3 and 4). The short *lag* time observed for the controls (containing 20% of damaged cells at time equal to 0) gives further support to this observation.

The higher Tg of GOS Biotempo with regard to GOS Cup Oligo H-70® and lactulose can explain their greater protectant capacity on the light of the vitrification hypothesis of preservation [38]. The different composition of GOS Biotempo and GOS Cup Oligo H-70® provides further

information for the interpretation of their protectant mechanisms. GOS Biotempo were obtained after removing mono and disaccharides from the whole mixtures of oligosaccharides, whereas GOS Cup Oligo H-70® not [35]. This results in: a) a higher content of DP3 and DP4 in GOS Biotempo and b) the presence of mono and dissaccharides in GOS Cup Oligo H-70®. Considering the water replacement hypothesis of preservation, one can conjecture that the higher content of larger oligosaccharides (DP3 and DP4) allows a stronger interaction with polar head groups [9]. Considering that vitrification is necessary but not sufficient to explain protection, this explanation seems to be plausible.

In this regard, Crowe *et al.* (1998) reported that the effect of sugars on depressing Tm can be determined by their size [9]. In fact, raffinose is more effective than trehalose and this sugar, more effective than glucose in decreasing Tm of dipalmitoyl phosphatidylcholine (DPPC) membranes [9]. Hincha *et al.* (2003) carried out a systematic study on the stabilization of egg phosphatidylcholine (EPC) membranes by raffinose family of sugars, including sucrose (DP2), raffinose (DP3), stachyose (DP4) and verbascose (DP5) [39]. They found that these sugars prevent fusion and leakage of EPC liposomes progressively better with increasing the DP. This stabilization was explained on the basis of the greater capacity of higher DP sugars to interact with lipid membranes (depressing Tm), and also to their higher Tg [39].

In GOS Cup Oligo H-70®, the presence of mono and disaccharides, together with oligosaccharides of greater DP, also has a vitrification effect, although lower than in GOS Biotempo [8,35,38]. Moreover, mono and disaccharides interact with membranes in a lesser extent than DP3 [9]. Cells dehydrated with lactulose were the most damaged after rehydration (76%; Figure 4). Lactulose is mainly known as prebiotic, and their physical chemical properties have never been addressed. According to the present results, the lower protective capacity could be explained on the basis of a lower vitrification capacity [38], but further studies are necessary to assess the interaction of lactulose with lipid membranes.

CONCLUSIONS

The impact of the obtained results can be considered from two main perspectives: a) the usefulness of NIR to provide complete information about bacterial physiology during growth kinetics; b) the efficiency of GOS and lactulose as bacterial protectants during dehydration.

The repair of bacterial damage resulting from dehydration is critical for starter fermentation. Registration of NIR spectra is as simple as turbidimetric determinations, but has the enormous advantage of providing information about physiological state of cells. In this sense, the possibility of following the evolution of viable cells in a quick and non-invasive way strongly supports the use of NIR for monitoring starter fermentation at an industrial level.

The protectant capacity of prebiotics like GOS and lactulose during freeze-drying has been addressed for the first time in the last years [7,8,37,38]. In this work, we provided an insight on their protection capacity against dehydration. The greater contribution of DP3 and DP4 in the composition of GOS Biotempo can explain their better protectant capacity on the basis of the vitrification hypothesis of preservation. Considering previous works carried out on related oligosaccharides, an interaction with polar head lipid membranes cannot be discharged, thus reinforcing the protectant capacity of GOS Biotempo [9,39].

Acknowledgments

This work was supported by the Argentinean Agency for the Scientific and Technological Promotion (Projects PICT/2008/145 and PICT/2011/0226), the Argentinean National Research Council (CONICET) (PIP2012-2014114-201101-00024), the Mexican PIFI/UAZ/2012-program, the Mexican National Research Council (CONACyT) [Project No. 153066 (2010)], and the bilateral project CONACyT-MINCyT (México, Argentina) (163687/208443; Mx11/01). Authors acknowledge D. Torres for providing GOS Biotempo and L. Delgadillo-Ruiz for technical assistance. AGZ and EET are members of the research career CONICET. MIS is doctoral fellow from CONICET.

Competing Interests: The authors declare that they have no competing interests.

REFERENCES

1. Tymczyszyn EE, Gómez-Zavaglia A, Disalvo EA. Effect of sugars and growth media on the dehydration of Lactobacillus delbrueckii ssp. bulgaricus. *J. Appl. Microbiol.* 2007;102:845-851.
2. Tymczyszyn EE, Díaz MR, Pataro A, Sandonato N, Gómez-Zavaglia A, Disalvo EA. Critical water activity for the preservation of *Lactobacillus bulgaricus* by vacuum drying *Int J Food Microbiol*. 2008;128:342-347.
3. Fonseca F, Béal C, Mihoub F, Marin M, Corrieu G. Improvement of cryopreservation of *Lactobacillus delbrueckii* subsp. *bulgaricus* CFL1 with additives displaying different protective effects. *Int Dairy J*. 2003;13:917-926.
4. Oldenhof H, Wolkers W, Fonseca F, Passot S, Marin M. Effect of sucrose and maltodextrin on the physical properties and survival of air-dried *Lactobacillus bulgaricus*: an *in situ* Fourier transform infrared spectroscopy study. *Biotechnol Prog*. 2005;21:885-892.
5. Santivarangkna C, Higl B, Foerst P. Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. *Food Microbiol.* 2008;25:429-441.
6. Golowczyc MA, Gerez CL, Silva J, Abraham AG, De Antoni GL, Teixeira PM. Survival of spray-dried *Lactobacillus kefir* is affected by different protectants and storage conditions. *Biotechnol Lett.* 2011;33:681-686.
7. Tymczyszyn EE, Gerbino E, Illanes A, Gómez-Zavaglia A. Galacto-oligosaccharides as protective molecules in the preservation of *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Cryobiology*. 2011;62:123-129.
8. Tymczyszyn EE, Sosa N, Gerbino E, Gómez-Zavaglia A, Schebor C. Effect of physical properties on the stability of *Lactobacillus bulgaricus* in a freeze-dried galacto-oligosaccharides matrix. *Int J Food Microbiol*. 2012;155:217-221.
9. Crowe JH, Carpenter JF, Crowe LM. The role of vitrification in anhysrobiosis. *Annu Rev Physiol*. 1998;60:73-103.
10. Crowe JW, Hoekstra FA, Crowe LM. Anydrobiosis. *Annu Rev Physiol.* 1992;54:570-599.
11. Al-Qadiri HM, Alami NI, Al-Holy MA, Lin M, Cavinato AG, Rasco BA. *J Rapid Meth Aut Mic.* 2008;16:73-89.

12. Harz M, Rösch P, Popp J. Vibrational spectroscopy-A powerful tool for the rapid identification of microbial cells at the single-cell level. *Cytometry*. 2009;75A:104-113.

13. Cervera AE, Petersen N, Lantz AE, Larsen A, Gernaey KV. Application of near-infrared spectroscopy for monitoring and control of cell culture and fermentation. *Biotechnol Prog*. 2009;*25*:1561-1581.

14. McClure FW. 204 years of near infrared technology: 1800-2003. *J Near Infrared Spec.* 2003;11:487-518.

15. Bonanno AS, Olinger JM, Griffiths PR. The origin of band positions and widths in near infrared spectra. In: Hildrum KI, Isaakson T, Naes T, Tandberg, A. Near infrared spectroscopy: bridging the gap between data analysis and NIR applications. London: Ellis Horwood. 1992:19-28.

16. Small GW. Chemometrics and near-infrared spectroscopy: avoiding the pitfalls. *Trend Anal Chem.* 2006;25:1057-1066.

17. Zhang L, Small GW, Arnold MA. Calibration standardization algorithm for partial least-squares regression: application to the determination of physiological levels of glucose by near-infrared spectroscopy. *Anal Chem*. 2002;74:4097-4108.

18. Vaccari G, Dosi E, Campi AL, González-Vera A, Matteuzzi R, Mantovani G. A near-infrarod spectroscopy technique for the control of fermentation processes: An application to lactic acid fermentation. *Biotechnol Bioeng*. 1993;43:913-917.

19. Sivakesava S, Irudayaraj J, Ali D. Simultaneous determination of multiple components in lactic acid fermentation using FT-MIR, NIR, and FT-Raman spectroscopic techniques. *Process Biochem*. 2001;37:371-378.

20. Rodriguez-Saona L, Khambaty F, Fry F, Calvey E. Rapid detection and identification of bacterial strains by Fourier transform near infrared spectroscopy. *J Agric Food Chem*. 2001;49:574-579.

21. Arnold SA, Gaensakoo R, Harvey LM, McNeil B. Use of at-line and *in situ* near infrared spectroscopy to monitor biomass in an industrial fed-batch *Escherichia coli* process. *Biotechnol Bioeng.* 2002;80:405-413.

22. Cimander C, Carlsson M, Mandenius CF. Sensor fusion for on-line monitoring of yoghurt fermentation. *J Biotechnol*. 2002;99:237-248.

23. Macedo MG, Laporte MF, Lacroix C. Quantification of exopolysaccharide, lactic acid, and lactose concentrations in culture broth by near-infrared spectroscopy. *J Agric Food Chem.* 2002;50:1774-1779.

24. Tamburini E, Vaccari G, Tosi S, Trilli A. Near-infrared spectroscopy: a tool for monitoring submerged fermentation processes using an immersion optical-fiber probe. *Appl Spectrosc*. 2003;57:132-138.

25. Tosi S, Rossi M, Tamburini E, Vaccari G, Amaretti A, Matteuzzi D. Assessment of in-line near infrared spectroscopy for continuous monitoring of fermentation processes. *Biotechnol Prog*. 2003;19:1816-1821.

26. Navrátil M, Cimander C, Mandenius CF. On-line multisensor monitoring of yogurt and filmjölk fermentations on production scale. *J Agric Food Chem.* 2004;52:415-420.

27. Saranwong S, Kawano S. System design for non-destructive near infrared analyses of chemical components and total aerobic bacteria count of raw milk. *J Near Infrared Spec*. 2008;16:389-398.

28. Saranwong S, Kawano S. Interpretation of near infrared calibration structure for determining the total aerobic bacteria count in raw milk: interaction between bacterial metabolites and water absorptions. *J Near Infrared Spec.* 2008;16:497-504.

29. Cámara-Martos F, Zurera-Cosano G, Moreno-Rojas R, García-Gimeno RM, Pérez-Rodríguez F. Identification and quantification of lactic acid bacteria in a water-based matrix with near-infrared spectroscopy and multivariate regression modeling. *Food Anal Method.* 2012;5:19-28.

30. Feng YZ, ElMasry G, Sun DW, Scannell AGM, Walsh D, Morcy N. Near-infrared hyperspectral imaging and partial least squares regression for rapid and reagentless determination of Enterobacteriaceae on chicken fillets. *Food Chem.* 2013;138:1829-1836.

31. Goldfeld M, Christensen J, Pollard D, Gibson ER, Olesberg JT, Koerperick EJ, Lanz K, Small GW, Arnold MA, Evans CE. Advanced near-infrared monitor for stable real-time measurement and control of *Pichia pastoris* bioprocesses. *Biotechnol Prog.* 2014;30:749-759.

32. Rault A, Béal C, Ghorbal S, Ogier JC, Bouix M. Multiparametric flow cytometry allows rapid assessment and comparison of lactic acid bacteria viability after freezing and during frozen storage. *Cryobiology*. 2007;55:35-43.

33. Gómez-Zavaglia A, Abraham A, Giorgieri S, De Antoni GL. Application of polyacrylamide gel electrophoresis and capillary gel electrophoresis to the analysis of *Lactobacillus delbrueckii* whole-cell proteins. *J Dairy Sci*. 1999;82:870-877.

34. De Man JO, Rogosa M, Sharpe ME. A medium for the cultivation of lactobacilli. *J Appl Bacteriol*. 1960;23:130-135.

35. Torres DPM, Bastos M, Goncalves MPF, Teixeira JA, Rodrigues LR. Water sorption and plasticization of an amorphous galacto-oligosaccharide mixture. *Carbohyd Polym*. 2011;83:831-835.

36. Hiraoka Y, Kimbara K. Rapid assessment of the physiological status of the polychlorinated biphenyl degrader *Comamonas testosteroni* TK102 by flow cytometry. *Appl Environ Microbiol.* 2002;68:2031-2035.

37. Golowczyc M, Vera C, Santos M, Guerrero C, Carasi P, Illanes A, Gómez-Zavaglia A, Tymczyszyn EE. Use of whey permeate containing in situ synthesized galacto-oligosaccharides for the growth and preservation of *Lactobacillus plantarum*. *J Dairy Res.* 2013;80:374-381.

38. Santos M, Gerbino E, Araujo-Andrade C, Tymczyszyn EE, Gomez-Zavaglia A. Stability of freeze-dried *Lactobacillus delbrueckii* subsp. *bulgaricus* in the presence of galacto-oligosaccharides and lactulose as determined by near infrared spectroscopy. *Food Res Int.* 2014;59:53-60.

39. Hincha DK, Zuther E, Heyer AG. The preservation of liposomes by raffinose family oligosaccharides during drying is mediated by effects on fusion and lipid phase transitions. *Biochim Biophys Acta.* 2003;1612:172-177.

CAPTIONS TO FIGURES

Figure 1: Growth kinetics of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333 dehydrated in the presence of GOS Biotempo (opened up triangles), GOS Cup Oligo-H70® (opened circles), and lactulose (opened down triangles). Controls: non-dehydrated microorganisms (full black squares); microorganisms dehydrated without sugar (full gray squares). Kinetics were followed by determining absorbance at 600 nm.

Figure 2: Growth kinetics of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333 followed by NIR. Arrows indicate the beginning (time equal to zero) and the end (time equal to 30 h) of the growth kinetics. (A) Control (non dehydrated microorganisms); (B) Microorganisms dehydrated without protectant; (C) Microorganisms dehydrated in the presence of GOS Biotempo; (D) Microorganisms dehydrated in the presence of GOS Cup Oligo H-70®; (E) Microorganisms dehydrated in the presence of lactulose.

Sripts I: normalized raw spectra; Scripts II: normalized second derivative spectra (inverted spectra are shown); Script III: normalized second derivative spectra in the 1350-1400 nm region (inverted spectra are shown).

Figure 3: Position of the inverted second derivative band at ~1370 nm as a function of the time of incubation. Microorganisms dehydrated with GOS Biotempo (opened up triangles), GOS Cup Oligo-H70® (opened circles), and lactulose (opened down triangles). Controls: non-dehydrated microorganisms (full black squares); microorganisms dehydrated without sugar (full gray squares). Circled symbols correspond to microorganisms in the late stationary phase.

Figure 4: Evolution of viable, damaged and dead microorganisms throughout the kinetics of growth. (A) Control (non dehydrated microorganisms); (B) Microorganisms dehydrated with GOS Biotempo; (C) Microorganisms dehydrated with GOS Cup Oligo H-70®; (D) Microorganisms dehydrated with lactulose; (E) Microorganisms dehydrated without

protectant; Full squares, opened triangles and opened circles denote viable, damaged and dead microorganisms, respectively.

Figure 5: Scores plot from the PCA carried out on the NIR spectra of *Lactobacillus delbrueckii* subsp. *bulgaricus* CIDCA 333 in different phases of growth. The spectra registered throughout all the kinetics of growth (controls and microorganisms dehydrated with the three sugars and without protectants) were considered for the analysis. Circles denote microorganisms in the *lag* phase, triangles, microorganisms in the *log* phase and squares, microorganisms in the stationary phase. Letters ***a***, ***b*** and ***c*** denote microorganisms in the *lag*, *log* and stationary phase of grow; letters ***c*** and ***d***, denote viable and damaged cells. The dash line separates groups ***c*** and ***d*** (viable and damaged cells).

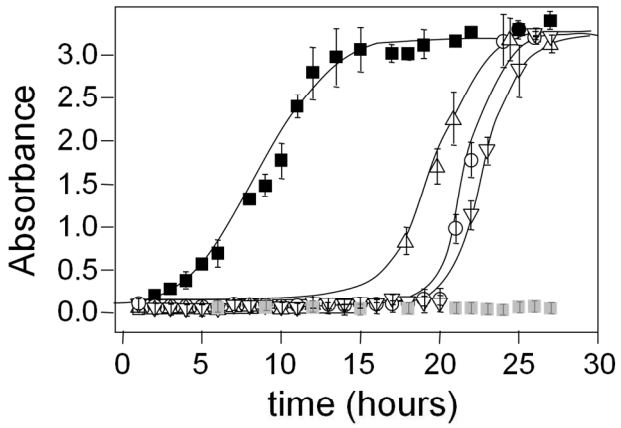


Figure 1

Figure 1
297x209mm (300 x 300 DPI)

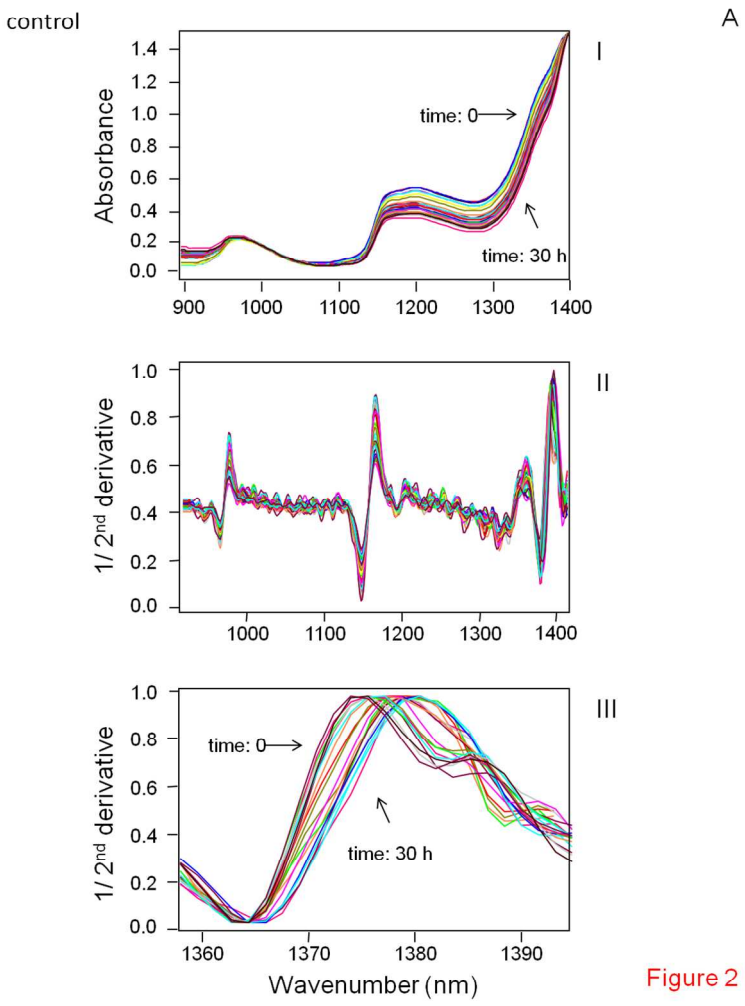


Figure 2

209x297mm (300 x 300 DPI)

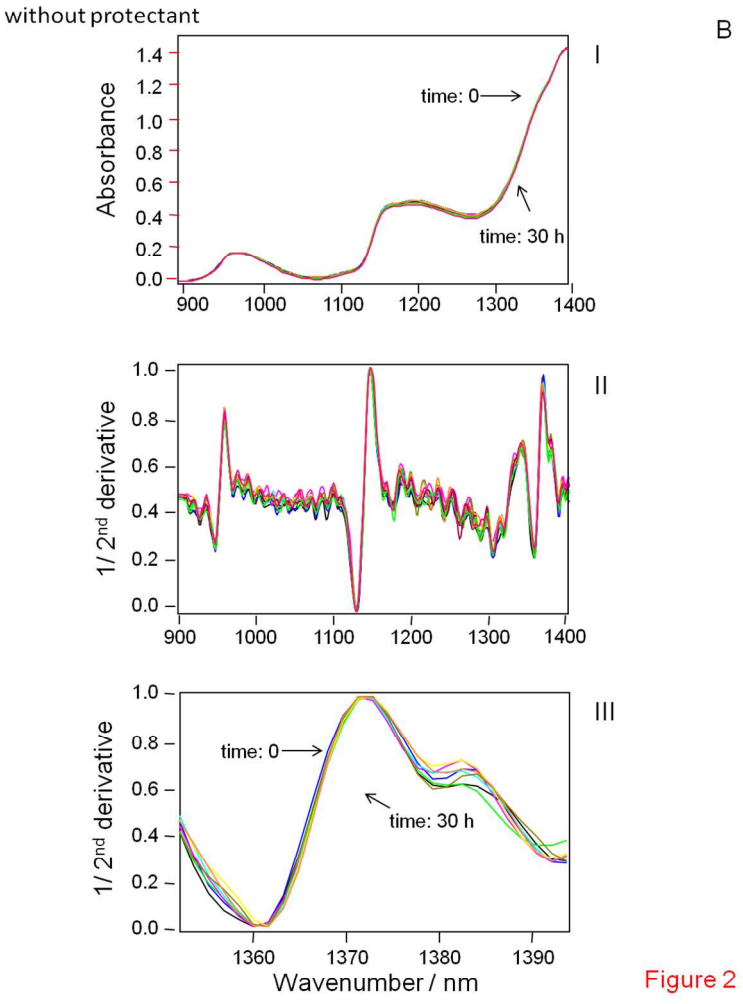


Figure 2

209x297mm (300 x 300 DPI)

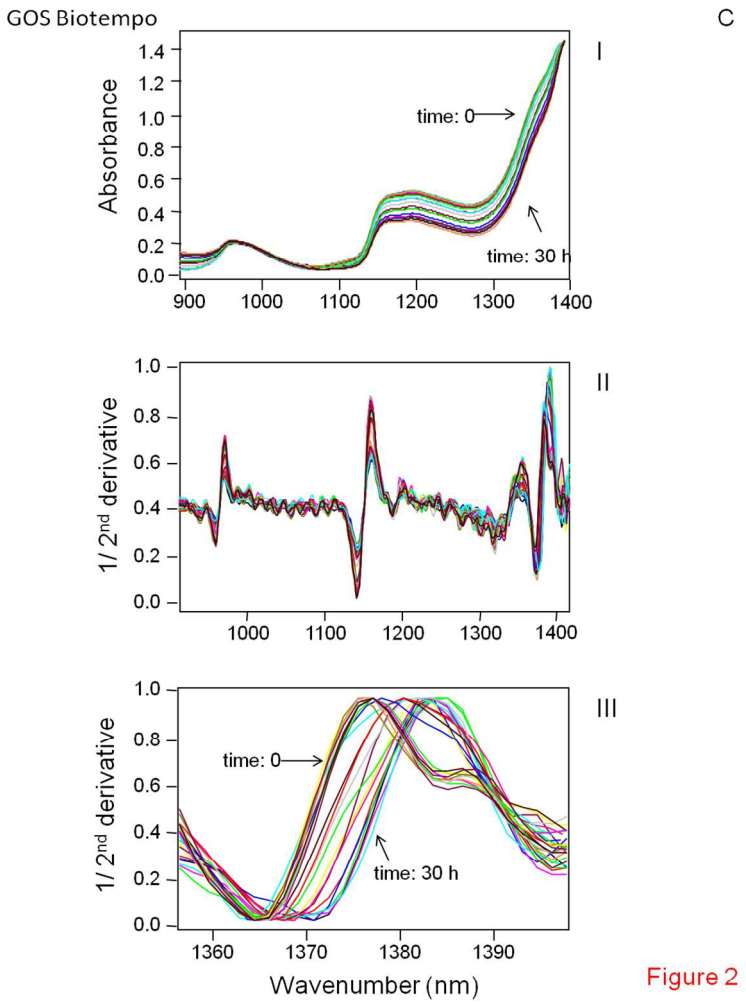


Figure 2

209x297mm (300 x 300 DPI)

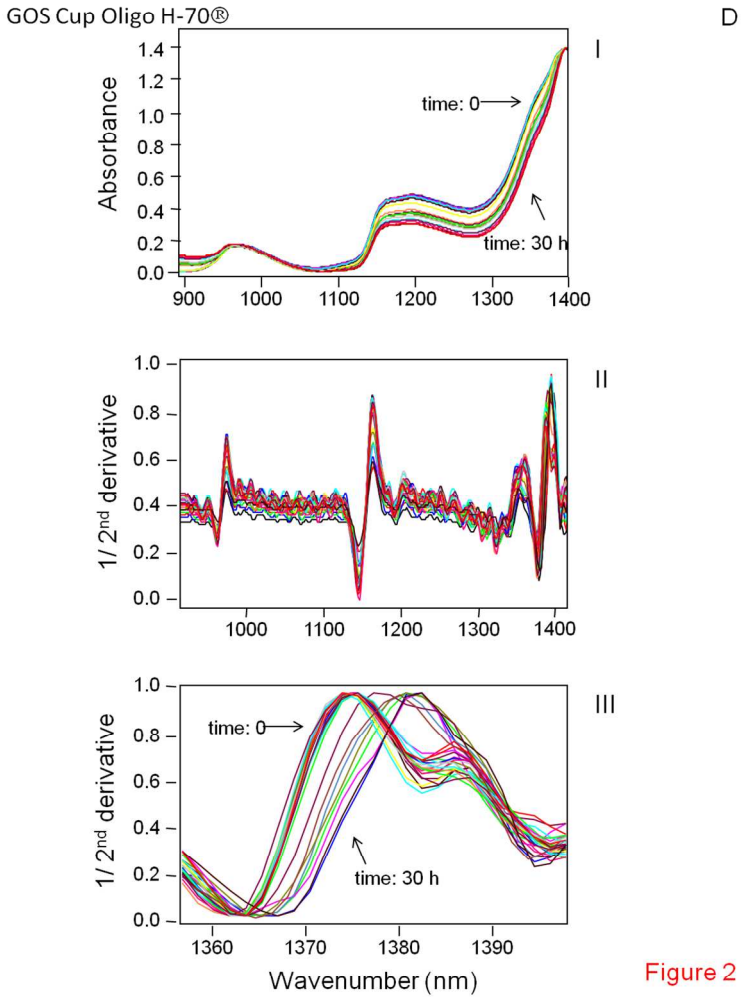


Figure 2

209x297mm (300 x 300 DPI)

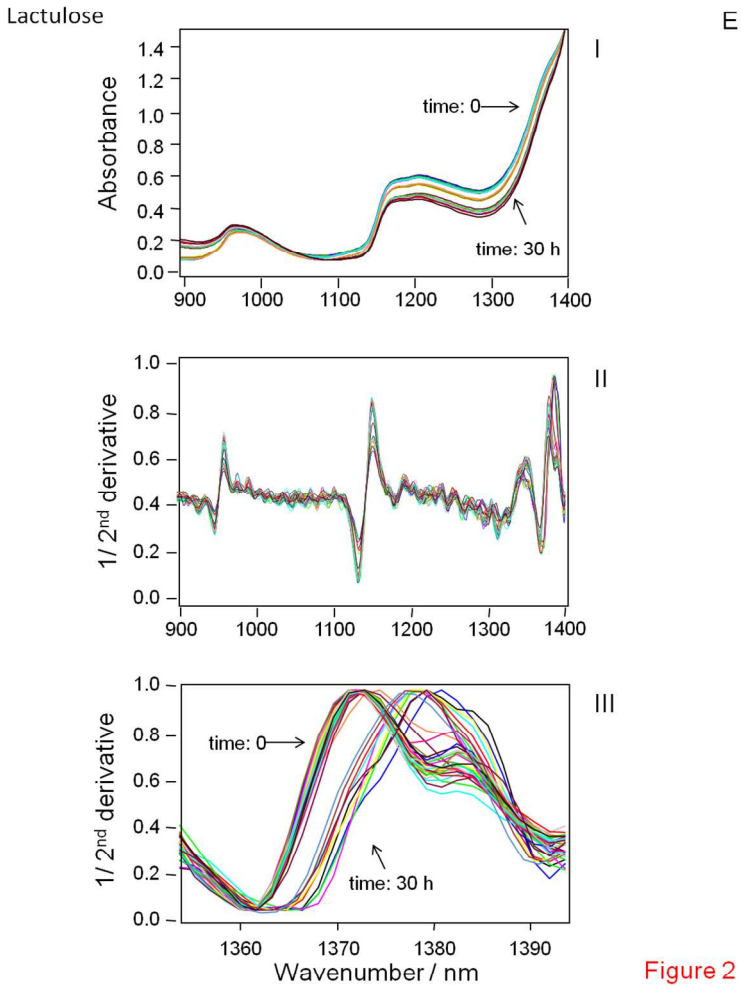


Figure 2

209x297mm (300 x 300 DPI)

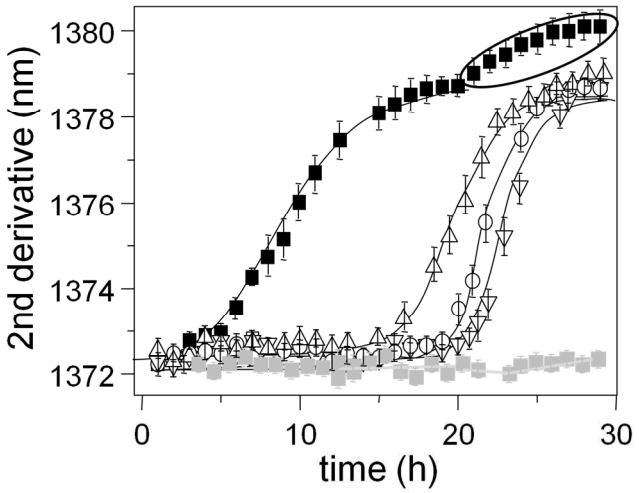
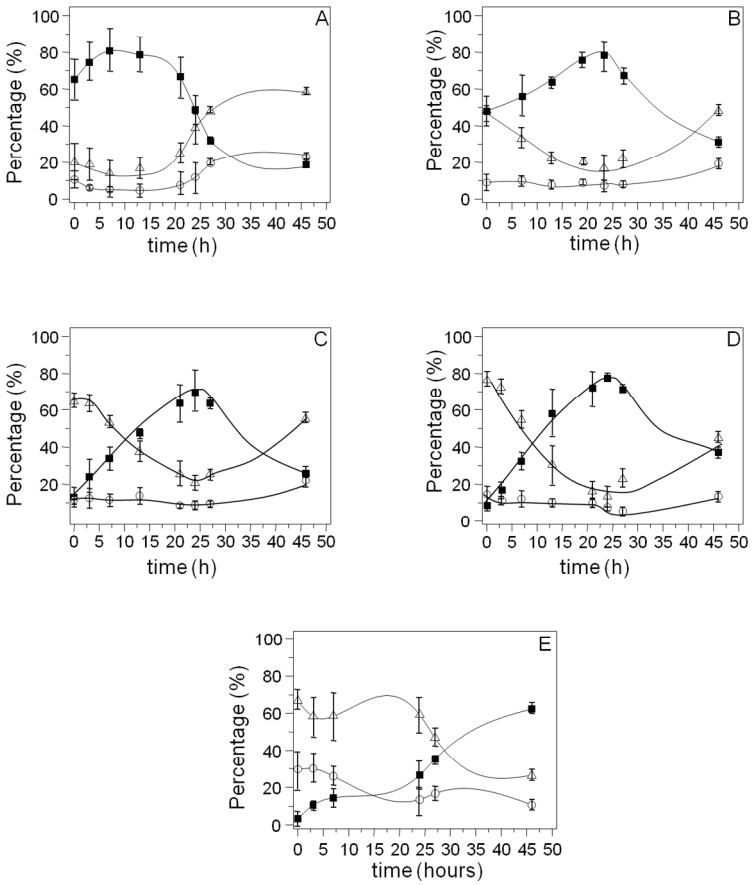


Figure 3

297x209mm (300 x 300 DPI)



209x297mm (300 x 300 DPI)

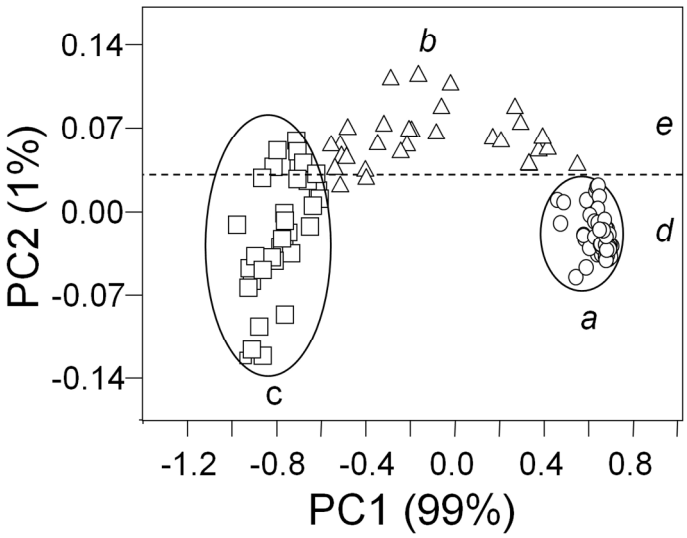


Figure 5

297x209mm (300 x 300 DPI)