Effect of innovative pre-treatments on the mitigation of acrylamide formation in potato chips

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Compared to untreated potato chips, yeast treatment promoted a reduction of AA formation mainly at the longest dipping time (15 min). PEF treatment followed by water dipping was the most effective in reducing AA for both the studied treatment times. The combination of PEF and yeast treatments led only a slight reduction of AA formation.

Keywords	Potato chips; Acrylamide; Pulsed electric fields; Aureobasidium pullulans yeast

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Highlights

– PEF and yeast were applied for the reduction of acrylamide content in potato chips.
– Yeast activity can promote the reduction of acrylamide content in potato chips.
– PEF treatment was the most effective in reducing acrylamide in potato chips.
– The pre-treatments slightly influenced the quality characteristics of potato chips.

Effect of innovative pre-treatments on the mitigation of acrylamide formation in potato chips

Authors

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Abstract

The aim of this research was to evaluate the reduction of acrylamide (AA) formation in potato chips applying innovative pre-treatments. Raw potato slices were subjected for 5 and 15 min to: dipping in water; dipping in *Aureobasidium pullulans* L1 yeast water suspension; dipping in water or in yeast water suspension after pulsed electric fields (PEF) in order to investigate a possible synergistic effect of pre-treatments. The raw potato samples were analysed for AA precursors and, after frying, for AA by using HPLC-MS/MS. In addition, the final potato chips main quality parameters were evaluated.

Compared to untreated potato chips, yeast treatment promoted a reduction of AA formation mainly at the longest dipping time (15 min). PEF treatment followed by water dipping was the

most effective in reducing AA for both the studied treatment times. The combination of PEF and yeast treatments led only a slight reduction of AA formation.

Industrial relevance

The Commission Regulation (EU) 2017/2158 has established new acrylamide (AA) benchmark levels in different foods due to its negative classification as "probably carcinogenic to human". For this reason, food industries are interested in developing different processing methods to reduce the AA formation and at the same time to maintain an acceptable quality of final products. Fried potatoes (French fries and chips) are the most vulnerable foods in terms of high content of AA formation, being rich in the main Maillard reaction substrates, such as asparagine and reducing sugars, and characterized by a high surface to volume ratio. Among the strategies recently suggested for the reduction of AA in potato chips, pulsed electric fields (PEF) and yeast pre-treatments are very promising, having the potentiality to reduce AA precursors in raw potato tissues. In this study the possibility to apply yeast and PEF pre-treatments on raw potato slices with suitable processing times for a possible industrial application were evaluated.

Keywords

Potato chips; Acrylamide; Pulsed electric fields; *Aureobasidium pullulans* yeast

Chemical compounds studied in this article

Acrylamide (PubChem CID: 6579); Asparagine (PubChem CID: 6267); Fructose (PubChem CID: 2723872); Glucose (PubChem CID: 5793).

1. Introduction

Acrylamide (AA) is an undesired food toxic compound classified as "probably carcinogenic to human" (group 2A), due to its neurotoxic and genotoxic proprieties on the basis of animal studies (IARC, 1994). In the last few years, authorities and regulations have been more and more restrictive concerning the maximum AA levels allowed in popular heat-treated foods and beverages (European Commission, 2017; Food Drink Europe, 2019; Palermo et al., 2016). The latest Commission Regulation (EU) 2017/2158, although not introducing a legal limit, contains stricter measures to reduce AA levels in different food groups and subgroups such as fried potato, bakery products and roasted coffee (European Commission, 2017).

AA is principally formed during food thermal processing (> 120 °C) by the reaction of free amino acids (e.g. asparagine) with reducing sugars (D-glucose and D-fructose) as part of the Maillard reactions, also known as non-enzymatic browning reactions (Stadler & Scholz, 2004). On the other hand, Maillard reactions result in physical and chemical changes, important for the development of some peculiar food characteristics like the desirable taste, colour and flavour (Medeiros Vinci, Mestdagh, & De Meulenaer, 2012; O'Connor, Fisk, Smith, & Melton, 2001). For this reason, the challenge for food industries and researches is to develop methods to reduce this toxicant, without changing the desired final properties.

Potato is the second major crop for human consumption in Europe and the fourth in the world, being considered an excellent source of energy, carbohydrates and nutritional fibres (Dourado et al., 2019). Nevertheless, fried potato products (French fries, chips, etc.) are highly susceptible to AA formation due to asparagine and reducing sugars content as well as the high temperatures applied during the frying process (Parker et al., 2012) and the high surface to volume ratio. Due to the large consumption of fried potato products worldwide, the reduction of undesirable AA, without compromising the sensory characteristics is essential (Dourado et al., 2019).

The principal strategies proposed to reduce AA in potato products are the selection of cultivar and storage conditions, the control of time and temperature of heat treatment, the application of different frying techniques (e.g. under vacuum), the use of asparaginase enzyme and hot water blanching as pre-treatments (Amrein et al., 2003; Foot, Haase, Grob, & Gondé, 2007; Medeiros Vinci et al., 2012; Pedreschi, Mariotti, Granby, & Risum, 2011; Romani, Bacchiocca, Rocculi, & Dalla Rosa, 2009). However, these strategies present different disadvantages such as long processing times, high costs, negative sensory modifications and difficult implementation for industrial scale. Therefore, it is necessary to find alternative methods to reduce the formation of AA in potato products.

Recently, the application of biocontrol agents as asparaginase producers (Di Francesco et al., 2019) and non-thermal treatments such as pulsed electric fields (PEF) (Genovese et al., 2019) were proven useful to reduce the AA precursors in the potato tissues and hence the subsequent AA formation. The metabolic activity of microorganisms could reduce the asparagine concentration through the activity of the asparaginase enzyme, which hydrolyses asparagine to aspartic acid and ammonia. Di Francesco et al. (2019) reported for the first time that the yeast *Aureobasidium pullulans* L1 strain can successfully assimilate asparagine in "Primura" var. potato homogenate after 30 min of contact, leading to a great decrease in the AA content in the final fried potato chips (- 85%). The yeast ability to produce enzymes has attracted considerable biotechnological interest because these hydrolytic enzymes have a potential commercial value in various industries (Deshpande, Rale, & Lynch, 1992). As demonstrated by Dunlop & Roon (1975), *A. pullulans* showed a hydrolytic cleavage of L-asparagine with intact whole cells. Generally, the liberation of enzymic activity from intact protoplast cells is considered the single conclusive proof for the existence of an extracellular enzyme (Dunlop & Roon, 1975), subsequently able to act on the potato tissue composition. Moreover, *A. pullulans* has demonstrated to be able to ferment sugars, among which sucrose covers an important role because proved as the carbon source for pullulan synthesis (Sheng, Tong, & Ma, 2016). Furthermore, An, Ma, Chang, & Xue (2017) showed how potato starch can promote *A. pullulans* enzyme production and pullulan biosynthesis. PEF technology consists in an electrical treatment of short time (from nanoseconds to milliseconds) that promotes the temporary or permanent loss of the semi-permeability of cell membranes in biological tissues, favouring mass transfer phenomena (Fincan & Dejmek, 2003; Puértolas, Koubaa, & Barba, 2016; Vorobiev & Lebovka, 2010). Recently, Genovese et al. (2019) described the possibility to reduce AA precursors in raw potatoes by the application of PEF followed by a 5 min water dipping, which led to a reduction of around 30% of AA content in fried potato chips.

The aim of present research was to evaluate the possibility to reduce AA in potato chips by applying for different times the above-mentioned innovative pre-treatments, *A. pullulans* L1 strain and PEF, alone or in combination.

The AA and its precursors (e.g. reducing sugar and asparagine) content, quality parameters such as moisture, oil content, colour and texture of potato chips were also evaluated.

2. Materials and methods

2.1. Potato

Fresh potato tubers (*Solanum tuberosum* cv Lady Claire) were purchased at the local market (Emilia-Romagna, Italy) and stored for a maximum of 15 days in the dark at 15 °C and 90% relative humidity (RH). Potato slices were prepared by reproducing as much as possible the main industrial production steps and conditions. Tubers were washed in running water, manually peeled and cut in slices of 1.5 ± 0.2 mm thickness by using an electric cutter machine mod. KAFPL0922N (CAD Italy, Italy). Potato slices were rinsed immediately after slicing for approximately 1 min in tap water (18 ± 2 °C) in order to eliminate part of the starch material on the surface.

2.2. Aureobasidium pullulans L1 strain

Aureobasidium pullulans L1 strain is a potential biocontrol agent mainly active against fruit postharvest pathogens, representing a promising alternative to common fungicides in the control of postharvest diseases (Di Francesco et al., 2018; Di Francesco, Ugolini, D'Aquino, Pagnotta, & Mari, 2017). The yeast was maintained on Nutrient Yeast Dextrose Agar (NYDA), consisting of nutrient broth 8 g, yeast extract 5 g, dextrose 10 g and technical agar 15 g (all from Oxoid, Basingstoke, UK) in 1 L of distilled water, and stored at 4 °C until use. Two days before the experiments, the yeast was cultured in 250 mL conical flasks, containing 50 mL of Nutrient Yeast Dextrose Broth (NYDB: NYDA without Agar) on a rotary shaker at 200 rpm for 48 h at 25 °C. Cell suspension was prepared by centrifugation of the liquid culture samples at 6,000 rpm for 10 min at 4 °C. Yeast cells were suddenly washed twice with sterile distilled water to remove the growth medium and suspended in sterile distilled water to reach a concentration of 10^8 cells/mL by using a hemocytometer. Yeast suspension was used for the pre-frying treatments and isothermal calorimetry assay.

2.3. Chemicals and reagents

Acrylamide (for molecular biology, ≥99% (HPLC), C_3H_5NO, molecular weight 71.08 g/mol, CAS No 79-06-1) and its precursors namely, L-asparagine (≥98% (HPLC), $C_4H_8N_2O_3$, molecular weight 132.12 g/mol, CAS No 70-47-3), D-(-)-fructose (≥99%, $C_6H_{12}O_6$, molecular weight 180.16 g/mol, CAS No 57-48-7) and D-(+)-glucose (analytical standard, $C_6H_{12}O_6$, molecular weight 180.16 g/mol, CAS No 50-99-7) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual stock solutions of AA, fructose and glucose, at a concentration of 1,000 mg/L, and asparagine, at 500 mg/L, were prepared by dissolving the pure standard compounds in water and storing them in glass-stoppered bottles at -18 °C. Afterwards, standard working solutions at various concentrations were prepared daily by appropriate dilution of the

158 stock solution with water. HPLC-grade acetonitrile was supplied by Sigma-Aldrich (Milano,
159 Italy). HPLC-grade formic acid (99%) was obtained from Merck (Darmstadt, Germany). C18
160 octadecyl silica sorbent was purchased from Phenomenex (Castelmaggiore, Bologna, Italy).
161 Deionized water (> 18 MΩ cm resistivity) was further purified using a Milli-Q SP Reagent
162 Water System (Millipore, Bedford, MA, USA). All solvents and solutions were filtered through
163 a 0.2 μm polyamide filter from Sartorius Stedim (Goettingen, Germany). Before HPLC
164 analysis, all samples were filtered with Phenex™ RC 4 mm 0.2 μm syringeless filter,
165 Phenomenex (Castelmaggiore, Bologna, Italy).
166
167 ***2.4. Pre-treatments***
168 The raw potato slices were subjected to different pre-treatments before frying, obtaining
169 different samples as showed in Figure 1. In detail, the raw potato slices were subjected to:
170 dipping in water for 5 and 15 min (samples named respectively W5 and W15); dipping in *A.*
171 *pullulans* L1 yeast water suspension for 5 and 15 min (Y5 and Y15 samples); dipping in water
172 after PEF in water (PW5 and PW15 samples) and dipping in yeast water suspension after PEF
173 in the same yeast suspension (PY5 and PY15 samples). Raw potato slices that have not
174 undergone any pre-treatment were considered as control sample (C).
175
176 *2.4.1. Aureobasidium pullulans L1 treatments*
177 The pre-treatment by using L1 strain suspension was carried out according to the patented
178 procedure No. WO2019058248A1 (authors: M. Mari, A. Di Francesco and L. Ugolini, *Alma*
179 *Mater Studiorum*, Bologna and CREA), with slight modifications. Each treatment was carried
180 out at room temperature (~25 °C) by dipping 80 g of potato slices in 200 mL of yeast suspension
181 concentrated 10^8 cells/mL for 5 (Y5) and 15 (Y15) min. The yeast untreated samples were
182 represented by 80 g of potato slices dipped in 200 mL of tap water at room temperature for the
183 same times (5 min: W5, 15 min: W15). After dipping, the potato slices were collected, rinsed
184 with tap water and carefully dried with absorbent paper. Each treatment for each sample (W5,
185 W15, Y5, Y15) was performed in triplicate.
186
187 *2.4.2. Pulsed electric fields (PEF) treatments*
188 PEF pre-treatments were performed using a lab-scale PEF unit delivering a maximum output
189 current and voltage of 60 A and 8 kV respectively mod. S-P7500 (Alintel, Italy). The generator
190 provides monopolar rectangular-shape pulses and adjustable pulse duration (5-20 μs), pulse
191 frequency (50-500 Hz) and total treatment time (1-600 s). The treatment chamber (50 mm

length × 50 mm width × 50 mm height) was a prototype built by Alintel (Italy) and consisted of two parallel stainless-steel electrodes (3 mm thick) with a 47 mm fixed gap. The outputs of the generator, tension and current, were monitored using a PC-oscilloscope mod. Picoscope 2204a (Pico Technology, UK). Raw potato slices (20 g) were treated at room temperature in tap water (100 mL) with an initial electrical conductivity of 536 ± 23.2 μS/cm at 25 °C (EC-meter mod. Basic 30, Crison, Spain) and delivering n = 1,000 pulses at fixed pulse width (10 ± 1 μs), frequency (100 Hz), time interval between pulses (10 ± 1 ms) and applying an electric field strength of 1.5 kV/cm, resulting in a specific energy input of 105 ± 5.5 kJ/kg, calculated according to Raso et al. (2016). For more details regarding the PEF-treatment protocol and the measured cell disintegration refer to Genovese et al. (2019).

The PEF treatment was repeated four times in order to obtain one batch (80 g) of treated product for each sample; temperature changes due to PEF treatment, measured with a temperature probe mod. TESTO 445 (Testo GmbH & Co, Milano, Italy), were negligible. After the PEF treatments, 80 g of potato slices were collected and left dipped in 200 mL of water for 5 (PW5) and 15 (PW15) min. After dipping, the potato slices were collected, rinsed with tap water and carefully dried with absorbent paper. Each dipping treatment was performed in triplicate for each sample (PW5, PW15), consequently the preliminary PEF treatment was repeated 12 times for each sample ((20 g x 4) x 3)).

2.4.3. Combination of treatments

The combination of PEF and yeast pre-treatments was performed filling the PEF treatment chamber with about 100 mL of yeast aqueous suspension (10^8 cells/mL) and 20 g of raw potato slices. The initial electrical conductivity of the yeast aqueous suspension measured using an electrical conductivity meter (EC-meter mod. Basic 30, Crison, Spain), was 536 ± 23.2 μS/cm at 25 °C (comparable with the tap water initial electrical conductivity used for PEF treatment in water). The selected PEF conditions and applied energy input were the same as mentioned in section 2.4.2. After the PEF treatments, 80 g of potato slices were collected and left immersed in 200 mL of the PEF-treated yeast suspension for 5 (PY5) and 15 (PY15) min. Subsequently, the potato slices were collected, rinsed with tap water and carefully dried with absorbent paper. Each dipping treatment in the PEF-treated yeast suspension was performed in triplicate for each sample (PY5, PY15); consequently the preliminary PEF treatment in the yeast aqueous suspension was repeated 12 times for each sample ((20 g x 4) x 3)).

2.5. Frying conditions

Untreated (C) and pre-treated potato slices were deep-fried in 6 L of high-oleic sunflower oil (potato to oil ratio 1:20 w/w) at 175 °C for 3 min, by using an electrical fryer mod. MFR280R (Fama Industrie, Italy). Temperatures of frying oil were monitored using K-type thermocouples sensors with an accuracy of 0.1 °C (Chromel/Alumel, Tersid Came, Italy), connected to a data logging system (mod. 9211A, National Instruments™, Texas).

2.6. Analytical determinations

2.6.1. Isothermal calorimetry

Isothermal calorimetry was used to evaluate the best combination of PEF pre-treatment and dipping in aqueous yeast suspension, by monitoring and determining the development of metabolic heat of the *A. pullulans* L1 yeast. The effect of PEF was investigated in different substrates. NYDB medium was used as ideal substrate for the yeast growth, while tap water and potato tissue in a 1:1 (w/w) ratio was used to simulate a real substrate. Moreover, because PEF could also increase the release of solutes from the potato tissue that could influence the yeast activity, tap water with potato tissue subjected to PEF was also considered. A schematic representation of the combination of samples and substrate is reported in Figure 2.

In each vial, 2 g of potato (raw or PEF-treated), 1 mL of yeast (10^8 cells/mL) (control and PEF-treated) and 2 mL of substrate (NYDB or tap water) were placed. Vials were sealed with Teflon caps and aluminium screw lids.

For each sample three replicas were performed. The extent of metabolic heat production by the yeast was measured continuously with a TAM Air isothermal calorimeter (TA Instruments/Thermometric, Sweden) with a sensitivity of ± 10 μW. This instrument contains eight twin calorimeters, each of which has its own reference to increase sensitivity and accuracy. As a reference, an ampoule with distilled water was used. The amount of distilled water was determined according to Panarese, Tylewicz, Santagapita, Rocculi, & Dalla Rosa (2012). Isothermal calorimetric measurements were performed at a constant temperature of 25 °C, chosen as the optimal one for yeast growth, for 48 hours. Baselines were recorded before and after each measurement for 30 min. The heat produced was continuously recorded with dedicated software (TAM Air assistant, TA Instruments/Thermometric, Sweden). The thermograms obtained were normalized for the weight of the sample.

2.6.2. HPLC-MS/MS analysis: quantification of asparagine, reducing sugars and acrylamide

The freeze-dried raw potatoes and potato chips samples were crumbled finely in mortar and 2 g of sample were weighed in a 50 mL conical flask. The extraction was performed with 20 mL of Milli-Q water firstly by 1 min of agitation in a vortex mixer and secondly by 10 min of ultrasound-assisted extraction at room temperature. After pouring into a 50 mL centrifuge plastic tube, the sample was centrifuged at 5,000 rpm for 10 min and the supernatant was collected and stored overnight at -18 °C to precipitate starch and facilitate the separation and removal of fat fraction. Later, the sample was thawed at room temperature, once again centrifuged at 5,000 rpm for 10 min and then 1 mL of water supernatant was transferred to a 1.5 mL microcentrifuge tube containing 100 mg of C18 sorbent. Before centrifugation at 13,300 rpm for 15 min, the sample was vortexed for 1 min. Finally, an aliquot of supernatant was collected and diluted 1:100 in mobile phase for asparagine analysis and 1:2 in acetonitrile for AA, fructose and glucose analysis. Before high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) injection, the diluted samples were filtered with a 0.2 µm syringeless filter.

The quantitation of AA, asparagine, fructose and glucose, is frequently performed by different methods and using various analytical instruments. For instance, sugars are often analysed by high performance liquid chromatography-refractive index detector (HPLC-RID) (Coelho et al., 2018; Vivanti, Finotti, & Friedman, 2006), asparagine using high performance liquid chromatography with fluorescence detection (HPLC-FD) (Amrein et al., 2003) and AA by high performance liquid chromatography-mass spectrometry (HPLC-MS) (Elmore et al., 2015; Zhou, Wang, Chen, & Zhang, 2015). Few works have reported the simultaneous analysis, in starchy foods, of these four molecules using a single approach (Nielsen, Granby, Hedegaard, & Skibsted, 2006). In the present research we introduced a simple method for simultaneous quantification of AA, asparagine, glucose and fructose by using HPLC-MS/MS.

The extraction process was optimized by taking the cue from previous data (Nielsen et al., 2006) with some modifications. Water was used as the extraction solvent for the high solubility of target molecules and a dispersive solid-phase extraction (DSPE) using C18 sorbent was chosen for sample clean-up, because was a simple and fast technique able to remove non-polar molecules which could act as interferences (Anastassiades, Lehotay, Štajnbaher, & Schenck, 2003).

HPLC-MS/MS studies were performed using an Agilent 1290 Infinity series and a Triple Quadrupole 6420 from Agilent Technology (Santa Clara, CA) equipped with an electrospray ionization (ESI) source operating in positive ionization mode. The HPLC-MS/MS parameters of each analyte were optimized in flow injection analysis (FIA) (1 µL of a 10 mg/L individual

standard solution) by using optimizer software (Agilent). The separation of target compounds was achieved on a Kinetex Hilic analytical column (100 mm × 4.6 mm i.d., particle size 2.6 µm) from Phenomenex (Torrance, CA, USA) preceded by a KrudKatcher ULTRA HPLC In-Line Filter (2.0 µm Depth Filter x 0.004in ID). The mobile phase for HPLC-MS/MS analysis was a mixture of 15% water (A) and 85% HPLC-grade acetonitrile (B), both with 0.1% formic acid. The separation was obtained by flowing at 0.8 mL/min with this gradient elution: isocratic condition until 2.5 min (85% B), 3.5 min (70% B), 5.5 min (70% B), 6.5 min (85% B) and then constant until the end of the run (15 min). All solvents and solutions were filtered through a 0.2 µm polyamide filter from Sartorius Stedim (Goettingen, Germany). The injection volume was 2 µL. The temperature of the column was 25 °C and the temperature of the drying gas in the ionization source was 350 °C. The gas flow was 12 L/min, the nebulizer pressure was 45 psi and the capillary voltage was 4,000 V. Detection was performed in the multiple reaction monitoring (MRM) mode. The MRM peak areas were integrated for quantification and the most abundant product ion was used for quantitation, and the rest of the product ions were used for qualification. The selected ion transitions and the mass spectrometer parameters are reported in Table 1.

As an example, Figure 3 shows the HPLC-MS/MS chromatogram of a standard mixture of the monitored compounds plotted as overlapped multiple reaction monitoring (MRM) transition of each compound. Results were expressed as µg/kg (dry matter) for AA and mg/kg (dry matter) for AA precursors.

2.6.3. Moisture

The moisture content was determined on all types of raw and fried potato samples by gravimetric method. The samples were dried to constant weight in a thermo-regulated laboratory stove (UF110, Memmert, Schwabach, Germany) at 70 °C (around 48 h) and 105 °C, (around 24 h) respectively for raw and fried potatoes. Results were expressed as percentage of water. The analysis was carried out in triplicate for each sample.

2.6.4. Oil content

The oil content of potato chips after frying was determined by Soxhlet extraction, performing the procedure with petroleum ether as a solvent at 60 °C for 3 h (AOAC, 1990). Oil content was reported as a percentage on dry matter. The analysis was carried out in triplicate for each sample.

2.6.5. Colour

The surface colour of whole potato chips was determined using a computer vision system (CVS). The samples were placed inside a dark chamber over a white background in controlled lighting conditions that consisted in four daylight fluorescent lamps (TL-D Deluxe, Natural Daylight, 18W/965, Philips, USA) with a colour temperature of 6500 K (D65 standard). The fluorescent tubes (60 cm long) were located 35 cm above the sample and inclined at an angle of 45°. The RGB images of the samples were acquired using a colour digital camera mod. D7000 (Nikon, Japan) equipped with 105 mm lens (mod. AF-S Micro Nikkor, Nikon, Japan), positioned vertically and connected to display and capture the images directly by the computer. For each sample, untreated and pre-treated, 10 images were captured on both sides of potato slices.

The pre-processing of RGB images and colour quantification in CIE $L^*a^*b^*$ scale was performed with ImageJ analysis software (NIH, USA). From numerical values of a^* (green-red) and b^* (yellow-blue) chromatic parameters, hue angle ($h°$) was calculated by the following equation (McGuire, 1992) and used to describe colour variations between samples:

$$h° = \tan^{-1}(b^*/a^*) \quad (1)$$

The colour measurements were carried out on the two surfaces of 5 potato chips for each sample.

2.6.6. Texture

The texture measurement on chips samples was performed at room temperature using a Texture Analyser mod. TA-HDi500 (Stable Micro System, UK) equipped with a 5 kg load cell and a spherical probe in stainless steel with a diameter of 6 mm at 1.0 mm/s test speed. The samples, selected on the basis of uniform size and shape, were placed on a support rig (HDP/CFS) and compressed for 3 mm distance. The acquired results were expressed as hardness, calculated by means of maximum force values, and as index of crispness, calculated by means of linear distance between the first and the last peaks registered (Tylewicz et al., 2019). Force vs distance curves were obtained from 12 potato chips for each sample.

2.7. Data analysis

The results were reported as mean value ± standard deviation of replications. The software STATISTICA 8.0 (Statsoft Inc., UK) was used for the statistical analysis of the data. Significant

differences between results were calculated by parametric analysis of variance (ANOVA) and Tukey multiple post-hoc comparison, with a significance level of 95% (p<0.05).

3. Results and Discussion

3.1. Preliminary tests on ideal substrate for the optimization of combined treatments

Isothermal calorimetry, that has been successfully used in the past to describe the growth ability of microorganisms (Braissant, Wirz, Beat, & Daniels, 2009), was employed to measure continuously and in real time the heat flow produced in different substrates by the yeast *A. pullulans* L1, untreated and subjected to the chosen PEF treatment protocol.

At first, the ideal growth substrate (NYDB) was used to evaluate the only effect of the selected PEF conditions on the yeast activity, in terms of heat produced. Figure 4A shows the heat flow, recorded at 25 °C during 35 h, produced by *A. pullulans* after PEF treatment (YPEF) compared to the untreated one (Y). The thermograms, as suggested by Morozova et al. (2017), are relative to the kinetic profile of the fermentative process. The heat flow signal has been considered as an index of the yeast growth rate. The shape of the signal suggests the presence of a series of consecutive processes that occur in the vial.

Initially, in both samples, a constant thermal power was recorded, of about $8.4 \cdot 10^{-4}$ and $2.0 \cdot 10^{-3}$ W/g for sample Y and YPEF respectively, indicating the lag phase. Typically, the lag phase corresponds to the period of time in which yeasts synthetize the enzymatic pool necessary for their catabolism during which the multiplication is neglectable (Morozova et al., 2017). After 10 h, the signal started to increase exponentially. This sudden change is due to the exponential growth phase of the yeast. The signal increased until reaching a maximum, in different times for the 2 samples, and then, because of various factors limiting the growth (e.g. reduced oxygen concentration in the head space, reduction of carbon and nitrogen sources in the growth medium, production of ethanol or increased pressure in the vial), the signal started decreasing, reaching almost a stationary phase, the extension of which depends on the ability of the yeast to survive (Morozova et al., 2017).

Table 2 reports the average values of the heat produced by the yeast according to the different conditions investigated, expressed as either total heat (integral of the heat flow) and slope of the curve related to the exponential growth phase. Results obtained suggest that PEF treatment had a positive effect on the yeast metabolism stimulating both its entity and rate. Different literature reports indicated that low intensity electrical fields (0.1-1 kV/cm) and short treatment times (µs-s) did not bring damage to the cell membranes functionality, but actually enhanced

microbial reactions and activities (Mattar et al., 2014; Schottroff, Krottenthaler, & Jaeger, 2017). According to these authors, low intensity PEF allows to improve enzymatic synthesis, frequency of cell division, probability of survival of daughter-cells, increases tolerance to inhibitors (e.g. ethanol) and yeasts fermentation ability. Moreover, electroporation induced by PEF can modify the cytoplasmic membrane and hence the nutrients transportation due to the formation of pores or the activation of transport proteins. However, such mechanisms have not been fully clarified yet and PEF effect depends also on other factors such as growth substrate, dimension and specific resistance of microbial cells (Mattar et al., 2014). In relation to the different susceptibility of different microorganisms and to the entity of PEF treatment applied, the cells can be in three different states: intact, dead or damaged (sub-lethal stress) (Wang et al., 2018).

Figure 4B shows the heat flow signals recorded at 25 °C during the yeast growth process, subjected to PEF (YPEF) compared to the control (Y), placed in a substrate simulating the real conditions, consisting of water and untreated potatoes or water and potatoes treated with PEF (PEF + Y and PEF + YPEF). In Table 2 the corresponding data of heat produced, and slope of the curve were reported. As expected, the extent of yeast growth in water-potato medium was significantly reduced compared to its optimal growth medium (NYDB). However, when placed in water-potato medium, no difference was observed for the yeast treated by PEF (PEF + YPEF) compared to its untreated control (PEF + Y) for both heat and slope produced. However, when the potato in the substrate was pre-treated by PEF (PEF+Y and PEF+YPEF), the heat produced almost doubled and the slope increased 5-6-fold in comparison to water-potato medium not treated with PEF (Y and YPEF). While total heat is an index of the metabolism entity, the slope indicates the rate of metabolic reactions. Our hypothesis is that PEF pre-treatment increased the membrane permeability of the potato tissue inducing a higher release of cell content into the water, that in turn became richer in nutrients exploitable by the yeast metabolism.

In addition, in the water-potato medium (real substrate) no differences in heat and slope were observed between PEF-treated (YPEF) and control (Y) yeast in both potato medium treated with PEF or not (Table 2). It seems that the positive effect of PEF on *A. pullulans* metabolism is visible only when the growth substrate is NYDB (Figure 4A), while in the case of the water-potato substrate, the limiting nutrient availability flattened the differences (Figure 4B).

Following these preliminary results, it was decided to PEF-treat the potato slices in the yeast water suspension in order to verify the combined effect of the two pre-treatments on AA formation.

3.2. Application on real substrate

3.2.1. Acrylamide and precursors analysis

The levels of AA in fried potato chips and the concentrations of AA precursors in untreated raw potato (control C) and in differently pre-treated raw potato samples are reported in Table 3. The AA concentration of untreated potato chips was 1,384.3 μg/kg, value that exceeds the reference level set by the Commission Regulation (UE) 2017/2158 (750 μg/kg). The untreated potato tubers were characterized by a glucose, fructose and asparagine content of 33.6 mg/kg, 6.8 mg/kg and 8,826.3 mg/kg respectively.

Each applied treatment, especially after 15 min of dipping, has led to a reduction of AA in final potato chips; however, not always proportional to the content of evaluated AA precursors.

Dipping in water (W5, W15) had a positive effect in reducing AA levels in fried potatoes due to the promotion of precursors release from the food matrix to the water for leaching effect. Potato samples dipped in water for 5 and 15 min (W5, W15) have presented a significant AA reduction respectively of 6.6% and 44.0%. The decrement of AA in these samples can be attributed to the reduction of glucose and fructose, while the asparagine content did not undergo any reduction. It is worth to note that the sample W15 resulted in AA content in compliance with the limit set by the EU Regulation. However, the concentration of AA precursors, and so the potential concentration of AA formation, is linked to intrinsic properties of the food matrix, i.e. potato cultivar, post-harvesting conditions. Therefore, AA levels in fried potatoes can be much higher than those measured in this study, explaining the necessity of assessing new AA mitigation measures suitable for industrial processing conditions.

The yeast treatment after 5 min (Y5) did not lead to a significant reduction in AA compared to the control (C) and to the water dipped sample for the same time (W5). On the other hand, after 15 min (Y15) a significant AA reduction of 51.1% was reached, highlighting that the yeast requires contact times greater than 5 min. Nevertheless, in comparison to the sample dipped in water for the same time (W15) the additional reduction after 15 min yeast treatment (Y15) was only 7%. This slight, but significant reduction of AA after 15 min yeast dipping could be attributed to enzymatic and metabolic activity of the yeast able to reduce the levels of glucose, fructose and asparagine. In a recent study with the same yeast strain, a reduction of AA in potato chips, equal to 83.7%, was obtained after a dipping of 30 min (Di Francesco et al., 2019). However, industrial application often requires shorter times.

The samples subjected to PEF treatment followed by dipping in water (PW) led to the highest AA reduction for each treatment time, equal to 35.9% and 58.7% respectively for 5 and 15 min.

The cell electroporation phenomena induced by the PEF treatment on raw potato slices resulted in a reduction of AA precursors leading to lower AA formation. This result confirms a recent study in which a significant reduction in the AA content (30%) was found in potato chips treated with PEF, compared to untreated samples (Genovese et al., 2019). Moreover, it is interesting to notice that, although precursors concentration was similar for Y15 and PW15 samples, AA levels were lower in PW15. This could be attributed to the possible modification of other molecules and pathways which can participate to AA formation. In fact, it is known that AA is mainly formed by Maillard reaction from asparagine and α-carbonyl sources such as reducing sugars, but other mechanisms can contribute to the final AA level. For example, the acrolein, which can be formed from lipid oxidation and degradation of amino acids, carbohydrate and protein, the acrylic acid derived from aspartic acid, and the β-alanine can participate to AA formation (Stadler & Studer, 2016).

The combination of PEF and yeast treatments (PY) resulted in a slight AA reduction (12.4% after 5 min and 13.8% after 15 min), similar to the results obtained with 5 min of dipping in water, but significantly lower compared to the reductions obtained with the singular pre-treatments (Y15, PW5 and PW15 samples). This result could be related to the direct effect of PEF on yeast cells that can notably influence their propriety and activity. In fact, as reported by Stirke et al. (2014) and Mattar et al. (2015) PEF can influence yeast cell viability, dimensions, and consequently their enzymatic activity. Although isothermal calorimetry results showed an increase of the overall yeast metabolic activity, the specific alterations of metabolic pathways induced by PEF are still unknown (Mattar et al., 2015). In order to elucidate how PEF can affect *A. pullulans* L1 strain activity additional experiments have to be performed in the future.

3.2.2. Qualitative analysis

The intense deep fry heat treatment is responsible for the development of non-enzymatic browning reaction (Maillard reaction) that can lead to undesired AA formation, but also to desired changes in physical attributes, such as colour, texture, flavour, moisture and oil content (Anese et al., 2009; Yang, Achaerandio, & Pujolà, 2016; Pedreschi, Moyano, Kaack, & Granby, 2005; Romani et al., 2009).

The main quality characteristics of fried potato samples were analysed in order to evaluate the possible effect of different AA mitigation pre-treatments applied (Table 4).

The development of a brown-gold colour during potato frying is one of the most important quality parameters demanded by customers (Medeiros Vinci et al., 2012). The use of the CVS allowed to evaluate the colour of whole potato chips surface. Example of RGB images of potato

chips samples untreated and differently treated are shown in Figure 5A, each RGB image was processed to extract the numerical CIE $L^*a^*b^*$ data (Figure 5B). The W15, Y15, PW5, PW15 and PY15 potato chips presented significant ($p<0.05$) higher L^* values compared to the other samples, highlighting a brighter colour (Table 4). The $h°$ values increased significantly for W15, Y15, PW5 and PW15 samples, underling a colour variation from the red-orange (dark) zone to the green-yellow (light) one compared to untreated sample (C).

The texture is another important sensorial characteristic of potato chips that should be monitored (Yee & Bussell, 2007). The various pre-treatments studied led to a slight and not always significant reduction of hardness and crispness index of potato chips compared with the untreated sample (C). To our knowledge, very few studies were carried out on the structural characteristics of the potatoes after similar pre-treatments. Zhou et al. (2015), using a different potato variety, found that the untreated potato samples were darker than yeast-treated ones characterized by a low AA content. However, no significant ($p>0.05$) differences in texture was found among the treated fried potato samples and untreated ones. The colour and texture results were also confirmed by the study of Di Francesco et al. (2019) who found that the potato untreated samples appeared darker in colour than yeast treated ones. Regarding the effect of PEF treatment, Ignat, Manzocco, Brunton, Nicoli, & Lyng (2015) observed significant differences only in L^* and a^* chromatic values for PEF-treated fried potato cubes, while the hardness was similar to the control sample. These results were confirmed also by Genovese et al. (2019) who found similar colour development during frying of PEF-treated potato slices. Moreover, the authors observed a slight and significant reduction of texture parameters in PEF-treated samples in comparison to the untreated one as in this case.

In the raw potato samples the water dipping treatments led to a significant moisture content increase for all treatment times (W5, W15) compared to untreated control sample (C). This increase is probably due to capillary and osmotic phenomena. No significant ($p>0.05$) differences were found between the moisture values of the samples subjected to dipping in water and in yeast water suspension for all treatment times. The raw samples subjected to PEF pre-treatment and subsequent dipping (PW5, PW15, and PY5, PY15) showed similar and lower moisture values than those treated only in dipping (W5, W15 and Y5, Y15). This result is probably explained by the increase of mass transfers from the sample to the dipping solution due to the modification of the cell membrane permeability induced by the PEF treatment. Similarly, in the fried potato chips, all PEF pre-treated samples showed a lower moisture content than the only dipping pre-treated ones. All fried potatoes subjected only to dipping pre-treatment presented moisture value not significantly different compared to the untreated one

(C). Nevertheless, the reduction of moisture due to frying was similar between the samples when compared with the respective raw samples and equal to 95-96%. This and the low values of standard deviation data demonstrate the uniformity of frying process conducted on the different samples and repetitions.

Regarding the oil content, all treatments (W, Y, PW and PY) promoted an increase of oil uptake (between 34-41%) compared to the fried untreated sample (C). This increase is probably due to the slightly higher water content in the treated raw samples, especially for W and Y ones. In fact, during frying the evaporation of the water leads to an absorption of frying oil (Aguilera & Gloria-Hernandez, 2000). However, in the PW and PY samples, subjected to PEF pre-treatment, which before frying showed the lowest water content compared to those subjected only to dipping (W and Y), the highest oil content was found (between 38-41%). This result suggests that in the PEF-treated samples the absorption of oil does not depend only on the initial water content, but also on the induced tissue electroporation phenomenon.

In the literature there are conflicting results regarding oil absorption in potato after PEF pre-treatments. According to Fauster et al. (2018), Ignat et al. (2015) and Janositz, Noack, & Knorr (2011), PEF treatment on potatoes led to a reduction in oil content of the final fried product in the shape of cubes or sticks (French fries). This result was ascribed to the transfer of water and intracellular substances to the potato surface due to the PEF-induced electroporation that, creating a barrier, reduced the oil uptake. This phenomenon is probably less evident in fried potato chips than in French fries. Moreover, in the present study the frying phase was not modulated as a function of the final reached moisture, while in the cited researches frying times were reduced after PEF pre-treatment and thus allowed to obtain a reduction in the oil absorption.

4. Conclusions

From the results obtained in this research work, the following conclusions can be drawn:

- the use of the yeast *A. pullulans* L1 strain in water suspension (patented procedure No. WO2019058248A1) as pre-treatment of potatoes to be fried as chips, was confirmed to be able to promote the reduction of AA in the finished product at dipping time longer than 5 minutes;
- the electroporation induced on raw potato slices by PEF treatment led to the highest reduction of AA formation in potato chips at each tested dipping time (5 and 15 min);
- the effect of the PEF treatment on the reduction of the AA formation in frying has been reduced when combined with dipping in yeast-water suspension for both tested dipping times;

- all the studied pre-treatments did not substantially influence the main final quality characteristics of potato chips.

The proposed strategies seem promising for the reduction of AA formation in fried potato products that will allow to comply with the current EU legislation, without causing detrimental effects on their final quality. However, further optimizations of tested pre-treatments are needed for industrial applications.

Moreover, the effect of PEF on the yeast activity should be further elucidated, in order to better exploit the yeast metabolism for AA reduction. Particularly, the time needed for the metabolic response of yeast to PEF treatment has to be carefully considered in order to optimize the studied pre-treatments.

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Figure 1. Scheme of processing pre-treatments steps studied for the acrylamide reduction in fried potato chips and corresponding samples codes.

Figure 2. Schematic representation of the combinations of sample and substrate evaluated by isothermal calorimetry as preliminary study to assess the possibility of combining PEF and yeast dipping pre-treatments (NYDB: dark blue; Water: light blue; Untreated Yeast: green; PEF-treated Yeast: light red; Untreated Potato: yellow; PEF-treated Potato: dark red).

Figure 3. HPLC-MS/MS chromatogram of a standard mixture of acrylamide, asparagine and sugars plotted as overlapped multiple reaction monitoring (MRM) transition of each compound.

Figure 4. Thermal profiles measured by isothermal calorimetry during growth of *A. pullulans* L1 strain subjected to PEF treatment, compared to its control growth in an ideal (A) and in real substrate (B).

Figure 5. Examples of RGB images of potato chips samples untreated and differently treated (A) and of images conversion from RGB into CIE *L*a*b** channels (B).

Fig. 1

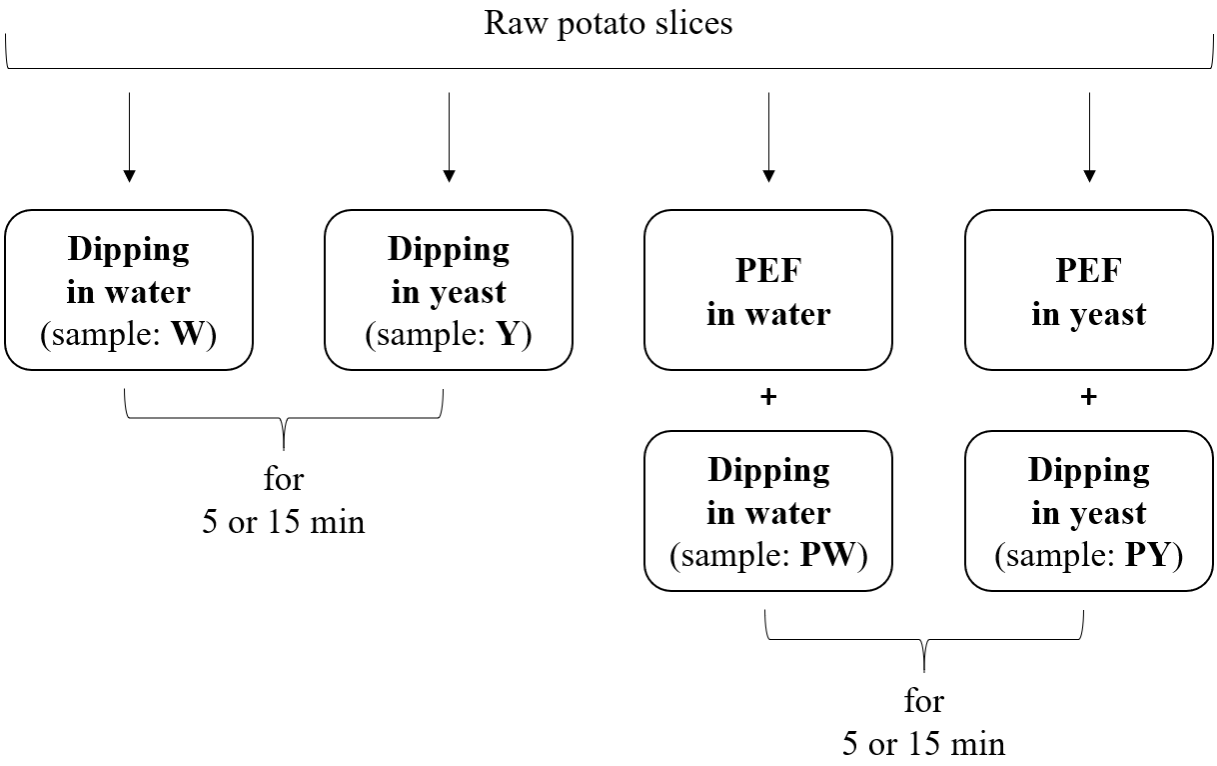
Raw potato slices
Dipping in water (sample: W)
Dipping in yeast (sample: Y)
PEF in water
PEF in yeast
+
+
Dipping in water (sample: PW)
Dipping in yeast (sample: PY)
for 5 or 15 min
for 5 or 15 min

Fig. 2

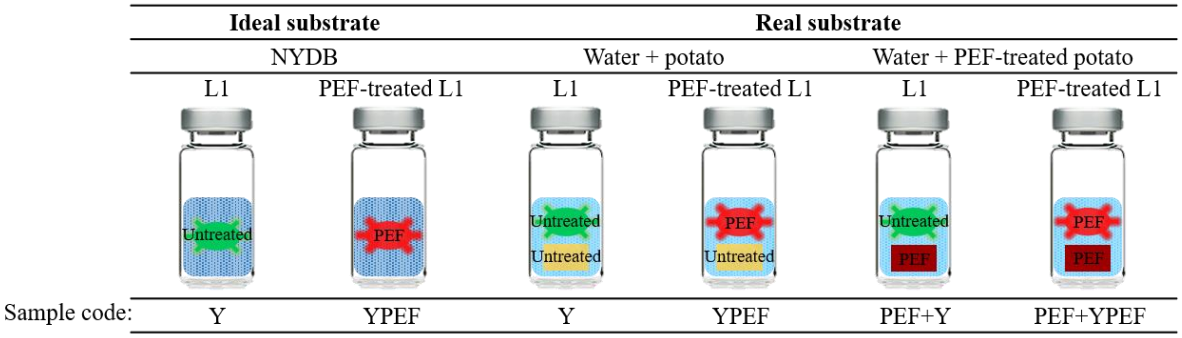
Ideal substrate
Real substrate
NYDB
Water + potato
Water + PEF-treated potato
L1
PEF-treated L1
L1
PEF-treated L1
L1
PEF-treated L1
Untreated
PEF
Untreated
Untreated
PEF
Untreated
Untreated
PEF
PEF
PEF
Sample code:
Y
YPEF
Y
YPEF
PEF+Y
PEF+YPEF

Fig. 3

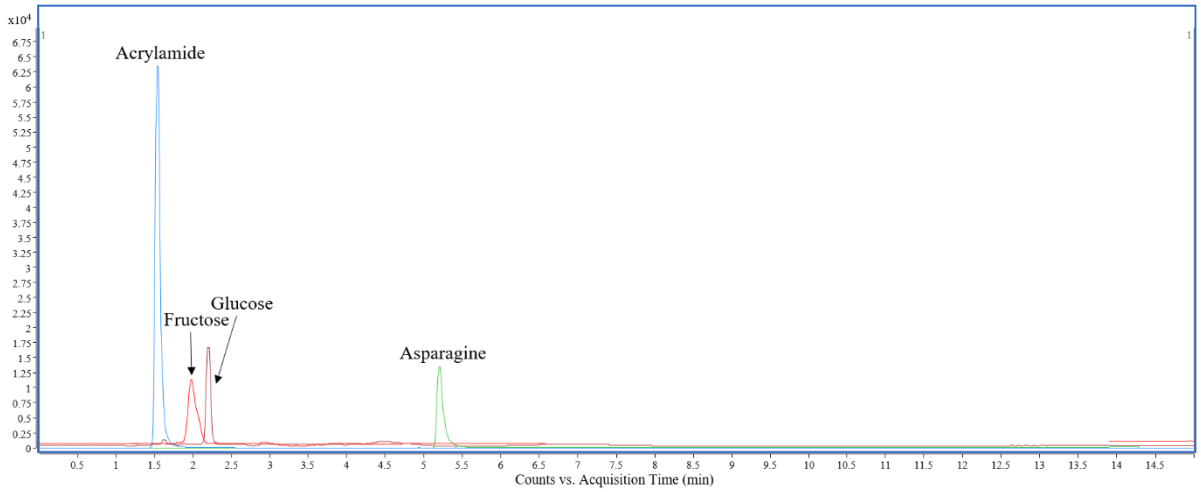
x10^4
Acrylamide
Glucose
Fructose
Asparagine
Counts vs. Acquisition Time (min)

Fig. 4

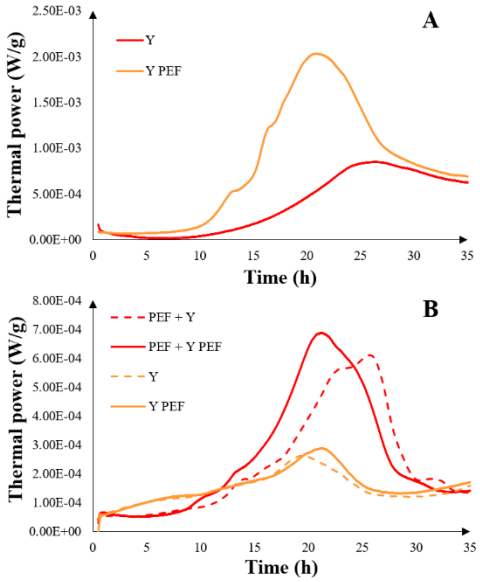
A
Y
Y PEF
Thermal power (W/g)
2.50E-03
2.00E-03
1.50E-03
1.00E-03
5.00E-04
0.00E+00
0
5
10
15
20
25
30
35
Time (h)
B
PEF + Y
PEF + Y PEF
Y
Y PEF
Thermal power (W/g)
8.00E-04
7.00E-04
6.00E-04
5.00E-04
4.00E-04
3.00E-04
2.00E-04
1.00E-04
0.00E+00
0
5
10
15
20
25
30
35
Time (h)

Fig. 5

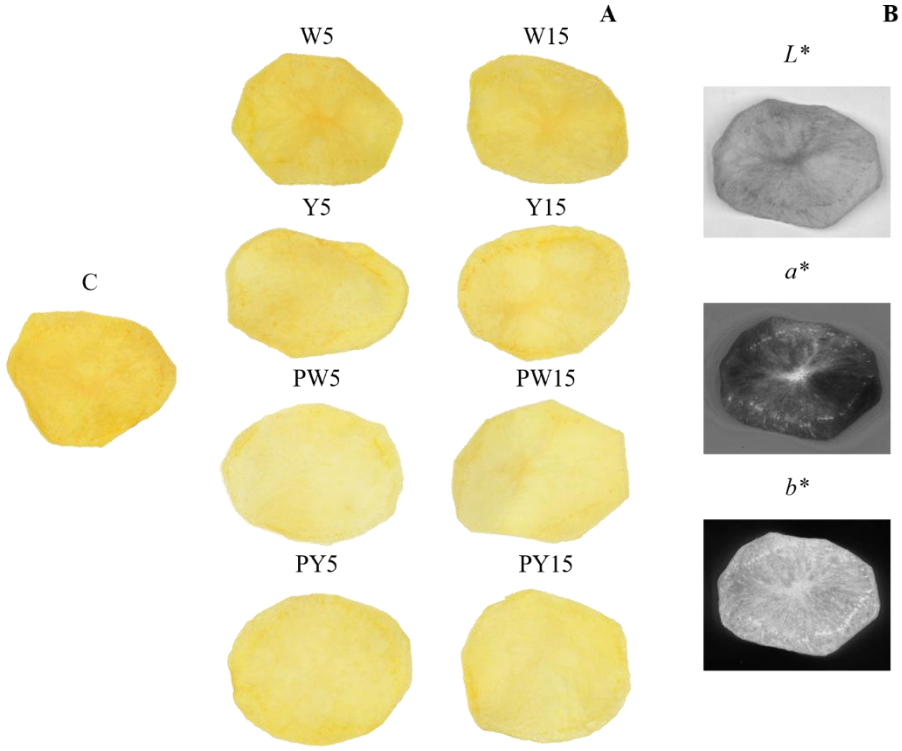

Table 1. HPLC-MS/MS acquisition parameters (MRM mode) adopted for the quantification of acrylamide, asparagine, and sugars.

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Fragmentor (V)	Collision Energy	Retention Time	Polarity
Acrylamide	72	55	45	8	1.52	Positive
Fructose	203	203	80	0	1.96	Positive
Glucose	203	203	80	0	2.18	Positive
Asparagine	133	74* 98	64	16 16	5.17	Positive

*This product ion was used for quantitative analysis; the rest for confirmatory analysis.

Table 2. Calorimetric parameters (heat and slope) measured during *A. pullulans* L1 growth in ideal (NYDB) and real (water+potato) substrates after PEF treatment compared to the control (Y: untreated yeast; YPEF: PEF-treated yeast; PEF: PEF-treated potato).

	Ideal substrate	
	Y	YPEF
Heat (J/g)	24.7 ± 2.8^{a}	45.8 ± 0.7^{b}
Slope (W/g·h)	$5.5 \pm 0.7 \cdot 10^{-5a}$	$2 \cdot 10^{-4} \pm 0.1^{b}$

	Real substrate			
	Y	YPEF	PEF + Y	PEF + YPEF
Heat (J/g)	17.9 ± 0.7^{a}	19.0 ± 0.9^{a}	31.6 ± 4.0^{b}	33.2 ± 2.3^{b}
Slope (W/g·h)	$1 \cdot 10^{-5} \pm 0.1^{a}$	$1.5 \pm 7 \cdot 10^{-5a}$	$6.5 \pm 4 \cdot 10^{-5b}$	$5.0 \pm 1 \cdot 10^{-5b}$

Different letters in the same line indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p<0.05$).

Table 3. Acrylamide levels in fried potato samples and acrylamide precursors contents in raw potato samples, untreated (C: control) and differently pre-treated at both 5 and 15 min (W: dipping in water; Y: dipping in yeast aqueous suspension; PW: PEF in water + dipping in water; PY: PEF in yeast aqueous suspension + dipping in yeast aqueous suspension).

Sample	Time (min)	Acrylamide (μg/kg)	Glucose (mg/kg)	Fructose (mg/kg)	Asparagine (mg/kg)
C		1384.3 ± 65.0^{a}	33.6 ± 6.8^{abc}	8.4 ± 1.0^{c}	8826.3 ± 576.0^{b}
W	5	1292.4 ± 96.2^{bc}	19.6 ± 0.4^{d}	6.9 ± 0.7^{d}	12952.0 ± 1341.9^{a}
	15	775.3 ± 81.5^{d}	29.50 ± 5.0^{c}	7.8 ± 1.1^{cd}	12036.4 ± 2142.5^{a}
Y	5	1375.9 ± 9.9^{ab}	39.5 ± 4.1^{a}	16.4 ± 0.7^{a}	12945.3 ± 441.7^{a}
	15	676.4 ± 42.3^{e}	18.6 ± 0.9^{d}	3.8 ± 0.2^{f}	5957.5 ± 135.6^{c}
PW	5	886.8 ± 9.9^{d}	36.7 ± 0.88^{ab}	5.0 ± 0.1^{ef}	4108.9 ± 571.3^{c}
	15	572.0 ± 8.8^{f}	17.8 ± 3.0^{d}	5.4 ± 0.4^{e}	5875.0 ± 695.9^{c}
PY	5	1211.9 ± 4.2^{c}	33.53 ± 1.2^{abc}	5.7 ± 0.2^{e}	4188.0 ± 69.9^{c}
	15	1193.2 ± 20.1^{c}	31.1 ± 1.2^{bc}	14.1 ± 0.5^{b}	4360.8 ± 87.9^{c}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p<0.05$).

Table 4. Colour parameters (*L**, *h*°), texture values (hardness, index of crispness), moisture and oil content of untreated (C: control) and treated potato chips (W: dipping in water; Y: dipping in yeast aqueous suspension; PW: PEF in water + dipping in water; PY: PEF in yeast aqueous suspension + dipping in yeast aqueous suspension).

		Raw potato	Fried potato					
Sample	**Time (min)**	**Moisture (%)**	**Moisture (%)**	***L****	***h*°**	**Hardness (N)**	**Index of crispness (linear distance)**	**Oil (%)**
C		74.1 ± 0.5^{b}	4.1 ± 0.1^{ab}	84.5 ± 1.1^{b}	93.2 ± 1.7^{ef}	3.9 ± 1.1^{a}	9.3 ± 2.6^{a}	30.3 ± 1.3^{d}
W	5	78.9 ± 0.4^{a}	4.2 ± 0.1^{a}	84.5 ± 1.2^{b}	94.6 ± 0.9^{de}	2.9 ± 0.4^{bc}	8.0 ± 1.7^{ab}	37.3 ± 0.1^{b}
	15	79.9 ± 0.8^{a}	4.1 ± 0.1^{ab}	87.1 ± 0.2^{a}	94.9 ± 0.6^{cd}	3.6 ± 0.8^{ab}	8.8 ± 2.6^{ab}	34.3 ± 0.6^{c}
Y	5	78.1 ± 0.7^{a}	4.0 ± 0.4^{ab}	84.3 ± 1.2^{b}	92.8 ± 0.8^{f}	3.7 ± 0.8^{ab}	9.7 ± 1.5^{a}	34.7 ± 0.8^{c}
	15	79.2 ± 0.2^{a}	3.7 ± 0.1^{b}	86.0 ± 1.3^{ab}	96.4 ± 1.1^{bc}	3.3 ± 0.6^{abc}	9.7 ± 2.4^{a}	33.5 ± 0.6^{c}
PW	5	74.5 ± 1.3^{b}	2.8 ± 0.1^{c}	86.1 ± 0.7^{ab}	98.9 ± 0.3^{a}	2.6 ± 0.7^{bc}	6.6 ± 2.1^{b}	41.3 ± 0.9^{a}
	15	75.6 ± 0.9^{b}	2.7 ± 0.3^{c}	86.4 ± 1.1^{ab}	97.8 ± 0.9^{ab}	3.1 ± 0.9^{bc}	9.1 ± 2.2^{a}	37.5 ± 0.8^{b}
PY	5	74.5 ± 0.9^{b}	2.5 ± 0.2^{c}	84.7 ± 1.3^{b}	93.5 ± 0.2^{def}	2.9 ± 0.7^{bc}	7.6 ± 2.9^{ab}	41.9 ± 0.8^{a}
	15	75.0 ± 0.4^{b}	2.5 ± 0.4^{c}	85.2 ± 0.8^{ab}	93.4 ± 0.2^{def}	3.0 ± 0.8^{bc}	8.8 ± 2.8^{ab}	38.4 ± 0.5^{b}

Different letters in the same columns indicate significant differences among samples according to Tukey multiple post-hoc comparison ($p<0.05$).

Declaration of interests

X The authors declare that they have no known competing financial interests or personal relationships
that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:
