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Wpływ elektroporacji na akumulację jonów żelaza w komórkach drożdży *Saccharomyces cerevisiae*

The effect of electroporation on the accumulation of iron ions in *Saccharomyces cerevisiae*

Rozprawa doktorska

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Składam serdeczne podziękowania
Pani dr hab. Monice Sujce, prof. uczelni
za opiekę naukową, poświęcony czas oraz niezliczoną ilość cennych rad.
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a w szczególności Pani Kierownik – ***prof. dr hab. Urszuli Pankiewicz****,*
która stworzyła sprzyjające warunki pracy,
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Spis treści

Streszczenie

Wpływ elektroporacji błony komórkowej na akumulację jonów żelaza w komórkach *Saccharomyces cerevisiae*

Celem pracy była ocena wpływu pulsacyjnego pola elektrycznego (PEF) na akumulację jonów żelaza w komórkach *Saccharomyces cerevisiae* oraz dobór warunków PEF optymalnych dla największego poboru tego pierwiastka. Jony żelaza akumulowały się najefektywniej, gdy ich źródłem był azotan (V) żelaza (III) oraz przy następujących parametrach PEF: napięcie 1500 V, szerokość impulsu 10 μs, czas działania PEF 20 min i liczba impulsów 1200, po 20 h hodowli. Drożdże wzbogacone w jony żelaza wykorzystano do produkcji podpłomyków, natomiast drożdże wzbogacone w jony żelaza oraz dodatkowo w witaminę B_{12} wykorzystano do produkcji płatków drożdżowych. W otrzymanych produktach oznaczano potencjalną biodostępność żelaza i witaminy B_{12}, zawartość podstawowych składników odżywczych oraz indeks glikemiczny. Przeprowadzono również ich ocenę sensoryczną. Potencjalna biodostępność żelaza z podpłomyków zawierających 385,8±4,12 mg żelaza w 100 g suchej masy wynosiła 10,83±0,94%. Ocena sensoryczna nie wykazała metalicznego posmaku w produkcie. W przypadku drożdży, z których otrzymano płatki drożdżowe, zastosowanie PEF skutkowało wyższą akumulacją żelaza i witaminy B_{12} odpowiednio o 140% i 100% w porównaniu z próbką, którą wzbogacono w wymienione składniki lecz bez PEF. Potencjalna biodostępność żelaza z płatków drożdżowych wyniosła 10,13%, a witaminy B_{12} 4,31%, a jakość tego produktu została oceniona przez panel sensoryczny jako dobra.

Słowa kluczowe: pulsacyjne pole elektryczne, drożdże, żelazo, witamina B_{12}, biodostępność

Abstract

Effect of cell membrane electroporation on the accumulation of iron ions in Saccharomyces cerevisiae cells

The aim of the study was to evaluate the impact of pulsed electric field (PEF) on the accumulation of iron ions in *Saccharomyces cerevisiae* cells and to select optimal PEF conditions for the highest uptake of this element. Iron ions accumulated most effectively when their source was ferric nitrate and with the following PEF parameters: voltage 1500 V, pulse width 10 μs, PEF operation time 20 min and number of pulses 1200, after 20 h of culture. Yeast enriched in iron ions was used for the production of flatbreads, while yeast enriched in iron ions and additionally in vitamin B_{12} was used for the production of yeast flakes. In products containing yeast enriched with iron ions and vitamin B_{12}, the potential bioavailability of iron and vitamin B_{12}, basic nutrients content and glycemic index were determined. A sensory evaluation of the tested products was also carried out. The potential bioavailability of iron from flatbreads containing 385.8±4.12 mg of iron per 100 g of dry weight was 10.83±0.94%. The sensory evaluation did not show metallic aftertaste in the product. In the case of yeast used for yeast flakes production, the use of PEF resulted in a higher accumulation of iron and vitamin B_{12} by 140% and 100%, respectively, compared to the sample enriched with these ingredients but without PEF. Potential bioavailability of iron from yeast flakes was 10.13%, and vitamin B_{12} 4.31%, and the sensory quality of this product was assessed as good.

Key words: pulsed electric field, yeast, iron, vitamin B_{12}, bioavailability

1. Wykaz publikacji wchodzących w skład rozprawy doktorskiej

PI. **Nowosad, K**., Sujka, M., Pankiewicz, U., Kowalski, R. (2021). The application of PEF technology in food processing and human nutrition. Journal of Food Science and Technology, 58(2), 397-411. (70 punktów MEiN, IF 3,117)

PII. **Nowosad, K**., Sujka, M., Pankiewicz, U., Miklavčič, D., Arczewska, M. (2021). Pulsed electric field (PEF) enhances iron uptake by the yeast *Saccharomyces cerevisiae*. Biomolecules, 11(6), 850. (100 punktów MEiN, IF 6,064)

PIII. **Nowosad, K**., Sujka, M. (2021). The use of iron-enriched yeast for the production of flatbread. Molecules, 26(17), 5204. (140 punktów MEiN, IF 4,927)

PIV. **Nowosad, K**., Sujka M., Wyrostek J. (2022). Preparation of yeast flakes enriched with iron and vitamin B12 using a pulsed electric field technology. Journal of Food Process Engineering, e14245. (100 punktów MEiN, IF 2,889)

Suma punktów za publikacje wchodzące w skład osiągnięcia naukowego według komunikatu MEiN obowiązującego w roku wydania pracy: **410**

Sumaryczny Impact Factor publikacji wchodzących w skład dzieła według listy Journal Citation Reports (JCR) zgodnie z rokiem opublikowania: **16,997**

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2. Uzasadnienie wyboru tematu pracy doktorskiej

Niedobór składników mineralnych oraz witamin stanowi jedną z przyczyn powikłań zdrowotnych. Witaminy i minerały odgrywają wiele ważnych ról w organizmie człowieka np. biorą udział w procesach wzrostu organizmu, a także poprawiają jego funkcjonowanie. Z tego względu konieczne jest utrzymywanie ich odpowiedniego poziomu w organizmie (Godswill i in., 2020).

Diety roślinne są popularne na całym świecie. Stosowanie diety wegetariańskiej lub wegańskiej wiąże się z wieloma korzyściami zdrowotnymi. Diety te dostarczają dużych ilości błonnika, kwasu foliowego, witamin C i E, potasu, magnezu oraz wielu fitozwiązków. Jednak wyeliminowanie z diety produktów pochodzenia zwierzęcego zwiększa ryzyko wystąpienia niedoborów żywieniowych, szczególnie żelaza oraz witaminy B_{12}. W związku z tym w przypadku niedoborów tych składników należałoby włączyć suplementację i wdrożyć odpowiednie nawyki żywieniowe (Weikert i in., 2020).

Żelazo jest niezbędnym składnikiem biorącym udział w wielu procesach biochemicznych np. w oddychaniu, wytwarzaniu energii, syntezie DNA (Camaschella, 2015), a także w regulacji wzrostu i różnicowania komórek (Melenovsky i in., 2017). Niedobór tego pierwiastka jest związany z wystąpieniem przewlekłej choroby nerek i niewydolności serca, nowotworów i nieswoistego zapalenia jelit (Cappellini i in., 2020). Dodatkowo długotrwałe narażenie organizmu na niedobór żelaza przyczynia się do wystąpienia anemii, która dotyka około jednej trzeciej światowej populacji. Jest to globalny problem zdrowia publicznego, na który szczególnie narażone są dzieci w wieku 0–5 lat, kobiety w wieku rozrodczym i kobiety w ciąży (Lopez i in., 2016).

Jednym ze sposobów leczenia niedoboru żelaza jest suplementacja doustna. Jednak stosowanie suplementów diety wiąże się wystąpieniem działań niepożądanych ze strony układu pokarmowego np. przy podawaniu żelaza na czczo mogą wystąpić nudności i ból w nadbrzuszu. Dodatkowo suplementy diety charakteryzują się niską przyswajalnością tego pierwiastka (Nowosad i Sujka, 2021). W celu profilaktyki i leczenia niedoborów żelaza stosuje się także fortyfikowanie żywności. Wzbogacenie żywności żelazem jest trudniejsze niż innymi składnikami odżywczymi np. soli kuchennej jodem czy oleju witaminą A, ponieważ żelazo wpływa na cechy sensoryczne produktu, zmieniając jego smak i barwę. Może również powodować utlenianie tłuszczu (Hurrell, 2002). Aby zapobiec tym niekorzystnym zmianom, można zastosować proces mikrokapsułkowania (Bishop i in., 1998). Jedną z metod mikrokapsułkowania jest akumulacja minerałów i witamin

w komórkach drożdży *Saccharomyces cerevisiae* (Pankiewicz i Jamroz, 2010; Pankiewicz i in., 2017; Nowosad i Sujka, 2021; Nowosad i in., 2022).

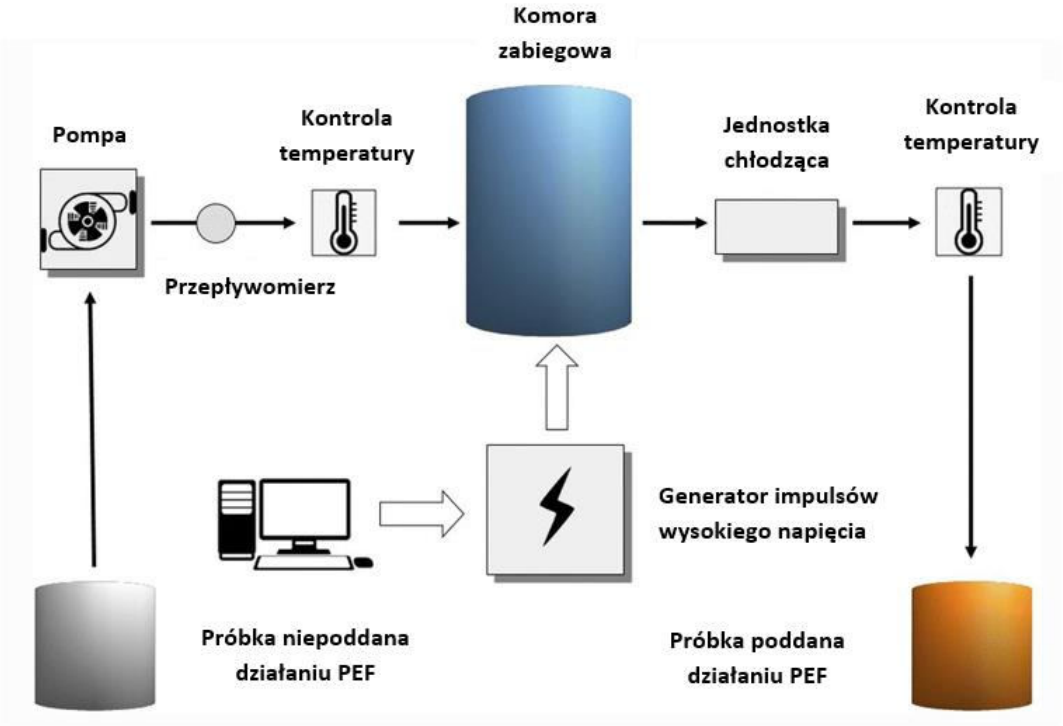
Błona komórkowa drożdży stanowi barierę między komórką, a środowiskiem zewnętrznym. Jednak w szczególnych okolicznościach bariera błonowa i selektywność transportu mogą zostać częściowo utracone, a cząsteczki mogą dostać się do cytoplazmy komórki. Zjawisko to występuje, gdy komórki są poddane działaniu pulsacyjnego pola elektrycznego (PEF) (Aronsson i in., 2001).

PEF to metoda wykorzystująca fale elektryczne o dużej amplitudzie napięcia. Do produktu umieszczonego między elektrodami w komorze dostarczane są krótkie impulsy elektryczne (od mikrosekund do milisekund) o wysokim napięciu (zwykle 10 - 80 kV/cm) (Deeth i in., 2007). W zależności od właściwości przetwarzanego produktu spożywczego i efektów, które mają być uzyskane, warunki procesu tj. natężenie pola elektrycznego (kV/cm), częstotliwość impulsu, szerokość impulsu, kształt impulsu i czas ekspozycji, można odpowiednio modyfikować. Na przykład zakres natężenia pola elektrycznego 0,1 - 1 kV/cm powoduje odwracalną permeabilizację komórek roślinnych, 0,5 - 3 kV/cm - nieodwracalną permeabilizację tkanki roślinnej i zwierzęcej, zaś 15 - 40 kV/cm - nieodwracalną permeabilizację komórek drobnoustrojów (Tsong, 1996).

Działanie PEF na komórki roślinne, zwierzęce lub drobnoustrojowe zaburza przejściowo lub trwale integralność błony komórkowej zwiększając jej przepuszczalność, jednak mechanizm działania PEF nie został jeszcze w pełni poznany. Zaproponowano kilka modeli teoretycznych, ale nadal nie ma dokładnych informacji o działaniu PEF na poziomie komórkowym (Soliva-Fortuny i in., 2009). Wyniki eksperymentów sugerują, że w błonie powstają hydrofilowe pory w wyniku przegrupowań składników błony, takich jak woda i lipidy, wywołanych działaniem długich i intensywnych impulsów elektrycznych (Weaver, 2003). Zgodnie z modelem zaproponowanym przez Zimmermanna (1986) ładunki o przeciwnych znakach są indukowane przez pole elektryczne na zewnętrznej i wewnętrznej powierzchni błony komórkowej. Gdy potencjał transbłonowy osiągnie wartość krytyczną, dwustronne przyciąganie ładunków prowadzi do powstania dużej liczby porów. Przepuszczalność błony komórkowej może wzrosnąć do poziomu umożliwiającego przedostanie się do wnętrza komórki np. cząsteczek DNA czy jonów metali. Gdy działanie pola elektrycznego ustaje, błona ulega uszczelnieniu, a komórki zatrzymują wprowadzone cząsteczki lub jony. Ponowne zamknięcie porów w błonie komórkowej może trwać od kilku sekund do kilku godzin, w zależności od temperatury otoczenia. Na przykład w 37°C pory błony zamykają się w kilka sekund, w 4°C w kilka minut, a gdy komórki są przetrzymywane

w lodzie - potrzeba na to kilku godzin. Gdy natężenie pola przekroczy wartość krytyczną, proces jest nieodwracalny i może prowadzić do zniszczenia komórek (Golzio i in., 2002).

Typowa jednostka PEF składa się z kilku podstawowych elementów: generatora impulsów wysokiego napięcia, komory obróbki, układu transportu cieczy, urządzeń kontrolno-monitorujących (Rys. 1). Pierwszy element dostarcza impulsy wysokiego napięcia o wymaganym kształcie, czasie trwania i natężeniu, które następnie są przekazywane do produktu umieszczonego między elektrodami w komorze obróbki. W zależności od rodzaju obrabianego produktu (stały, płynny, półpłynny) komory obróbki można podzielić na komory obróbki okresowej i komory obróbki ciągłej. Procesem steruje komputer centralny, który służy do ustawiania parametrów, sterowania pracą pompy oraz zbierania danych z sond umieszczonych wewnątrz komory (Barbosa-Canovas i in., 2004).



Rysunek 1. Typowa jednostka PEF stosowana w przetwórstwie spożywczym (Nowosad i in., 2021)

Pulsacyjne pole elektryczne można wykorzystać do zwiększenia akumulacji składników mineralnych i witamin w komórkach drożdży (Pankiewicz i in., 2017; Nowosad i in., 2021; Nowosad i in., 2022). W ten sposób tworzą się kompleksy metali z białkami zwane metaloproteinami (lub biopleksami), które są wysoko przyswajalne przez organizm człowieka (Liu i in., 2002). Biomasa drożdży wzbogacona w żelazo oraz witaminę B_{12} za pomocą pulsacyjnego pola elektrycznego może potencjalnie stanowić dodatkowe źródło tych składników w diecie, zwłaszcza dla wegan i wegetarian.

W literaturze naukowej wciąż niewiele jest opublikowanych badań dotyczących akumulacji jonów metali oraz witamin w komórkach mikroorganizmów w warunkach działania PEF, a także wykorzystania drożdży wzbogaconych w jony żelaza oraz witaminę B_{12} za pomocą PEF do produkcji żywności potencjalnie funkcjonalnej.

3. Cel i zakres pracy doktorskiej oraz hipotezy badawcze

Postawionym do rozwiązania problemem naukowym było zastosowanie pulsacyjnego pola elektrycznego do wzbogacania komórek drożdży w jony żelaza w celu wykorzystania ich do produkcji żywności funkcjonalnej. Celem niniejszej pracy była ocena wpływu parametrów pulsacyjnego pola elektrycznego na akumulację jonów żelaza w komórkach *Saccharomyces cerevisiae*.

Hipotezy badawcze sformułowane w niniejszej pracy są następujące:

1. Zastosowanie pulsacyjnego pola elektrycznego powoduje wzrost akumulacji jonów żelaza w komórkach drożdży.
2. Optymalizacja poszczególnych parametrów PEF przyczynia się do zwiększenia akumulacji jonów żelaza w komórkach drożdży.
3. Zastosowane parametry PEF nie wpływają istotnie na żywotność i przyrost biomasy drożdży.
4. Wzbogacenie komórek drożdży w jony żelaza z zastosowaniem PEF nie wpływa na ich aktywność fermentacyjną.
5. Komórki drożdży wzbogacone w jony żelaza można wykorzystać do produkcji żywności przeznaczonej dla osób zagrożonych niedoborem tego pierwiastka w diecie.

4. Materiał i metody badawcze

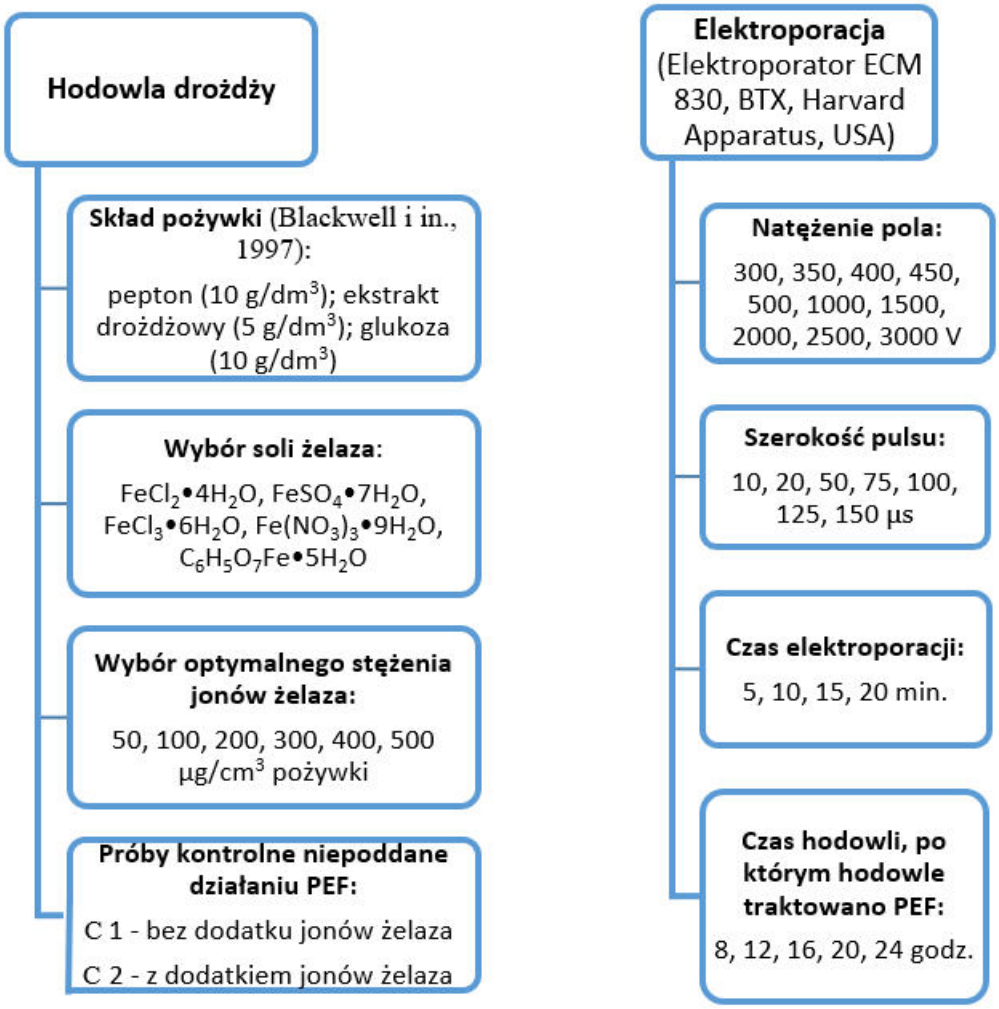
Materiał badawczy stanowił szczep *Saccharomyces cerevisiae* 11 B1 pochodzący z kolekcji Katedry Biotechnologii, Mikrobiologii i Żywienia Człowieka Uniwersytetu Przyrodniczego w Lublinie.

Zadania badawcze obejmowały:

- wybór soli żelaza o najwyższej akumulacji jonów żelaza w *Saccharomyces cerevisiae* przy wyjściowych parametrach PEF: napięcie 1500 V; szerokość pulsu 20 μs, czas elektroporacji 10 minut, czas hodowli, po którym komórki drożdży poddano działaniu PEF - 20 h;
- uzyskanie maksymalnej akumulacji jonów żelaza w *Saccharomyces cerevisiae* w warunkach traktowania hodowli pulsacyjnym polem elektrycznym:
  - ✓ elektroporacja błon komórek drożdży przy wzrastającym stężeniu jonów żelaza,
  - ✓ optymalizacja parametrów PEF (napięcie, szerokość pulsu, czas elektroporacji oraz czas hodowli, po którym komórki drożdży zostaną poddane elektroporacji),
  - ✓ określenie zmian żywotności komórek, wydajności biomasy oraz właściwości fermentacyjnych po traktowaniu hodowli PEF oraz przy wzrastającym stężeniu jonów metali;
- analizę rozmieszczenia jonów żelaza w komórce (mikroskopia fluorescencyjna oraz ATR- FTIR);
- otrzymywanie produktów z drożdży wzbogaconych w żelazo oraz w żelazo i witaminę B_{12} przy optymalnych parametrach PEF oraz zbadanie potencjalnej biodostępności żelaza oraz witaminy B_{12} metodą in vitro;
- analizę sensoryczną produktów otrzymanych z dodatkiem drożdży wzbogaconych w żelazo oraz w żelazo i witaminę B_{12}, analiza podstawowego składu odżywczego produktów, ich indeksu glikemicznego oraz właściwości przeciwutleniających.

Do realizacji powyższych zadań wykorzystano następujące metody badawcze:

1. Hodowle drożdży i poddanie ich działaniu pulsacyjnego pola elektrycznego wykonano wg schematu przedstawionego na rysunku 2.



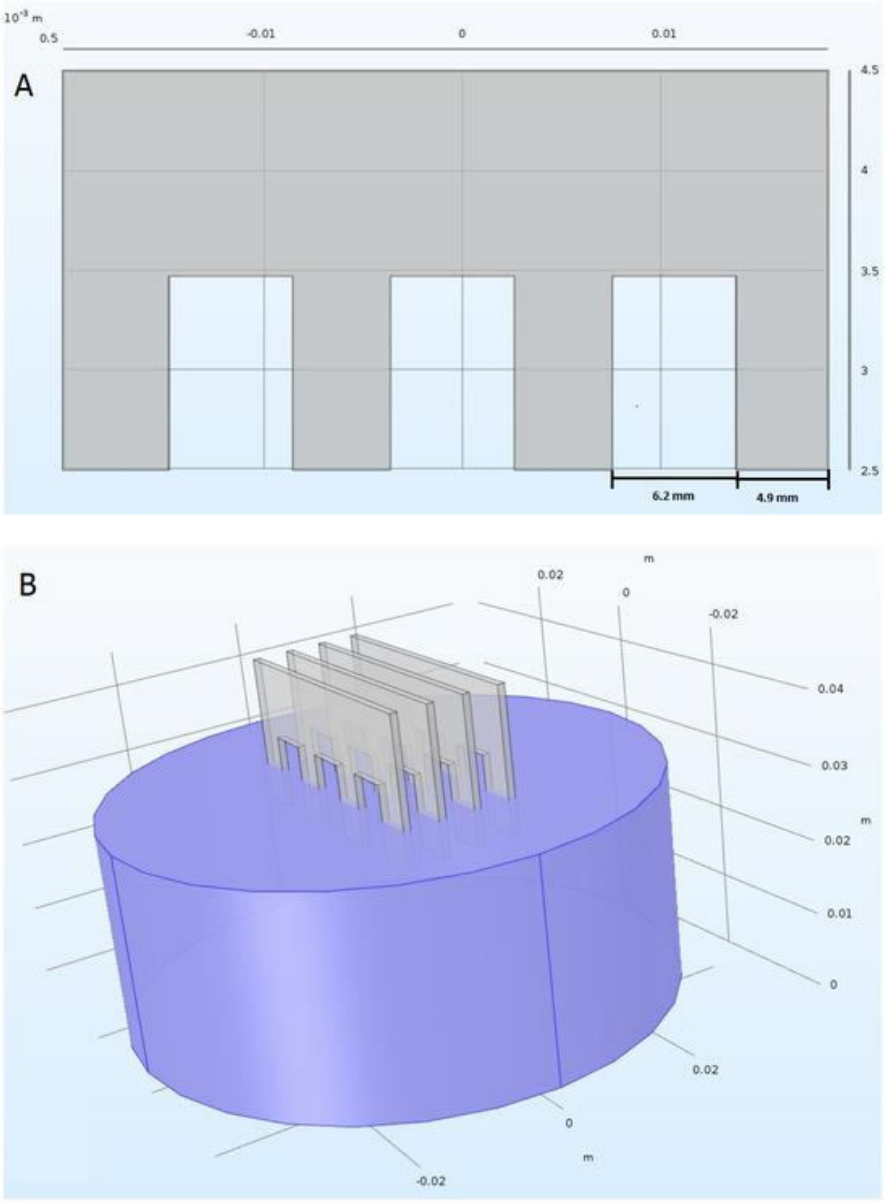
Rysunek 2. Schemat hodowli biomasy drożdży oraz schemat elektroporacji.

Parametry procesu optymalizowano utrzymując wszystkie czynniki na stałym poziomie

z wyjątkiem badanego (metoda OFAT, ang. *one factor at a time*). Doboru wartości parametrów dokonano na podstawie wcześniejszych badań Pankiewicz i Jamroz (2010). Zoptymalizowano następujące parametry: stężenie jonów żelaza, napięcie PEF, szerokość pulsu, czas elektroporacji oraz czas hodowli, po którym stosowano PEF. Sól żelaza wybrano w osobnym eksperymencie.

Hodowle *S. cerevisiae* prowadzono w kolbach o poj. 500 ml z ciągłym mieszaniem przez 20 godzin, a następnie traktowano PEF przy użyciu jednobiegunowego generatora fali prostokątnej ECM 830 (BTX Harvard Apparatus, Holliston, MA, USA). Hodowlę o objętości 100 ml umieszczono w komorze do obróbki PEF składającej się ze zlewki (300 ml) i czterech równoległych elektrod ze stali nierdzewnej o powierzchni równej 4 cm^2, ustawionych naprzeciw siebie w odległości 5,1 mm (rys. 3) zamontowane na zdejmowanej

pokrywie. Przewodność pożywki hodowlanej wynosiła 2,6 mS/cm (konduktometr CC-505, Elmetron, Zabrze, Polska), a częstotliwość dostarczania impulsów wynosiła 1 Hz. Podczas dostarczania impulsów roztwór mieszano za pomocą obracającego się magnesu (100 obrotów na minutę), aby uniknąć sedymentacji komórek. Elektrody zanurzono w roztworze na około 7,4 mm. Temperaturę monitorowano podczas działania PEF.



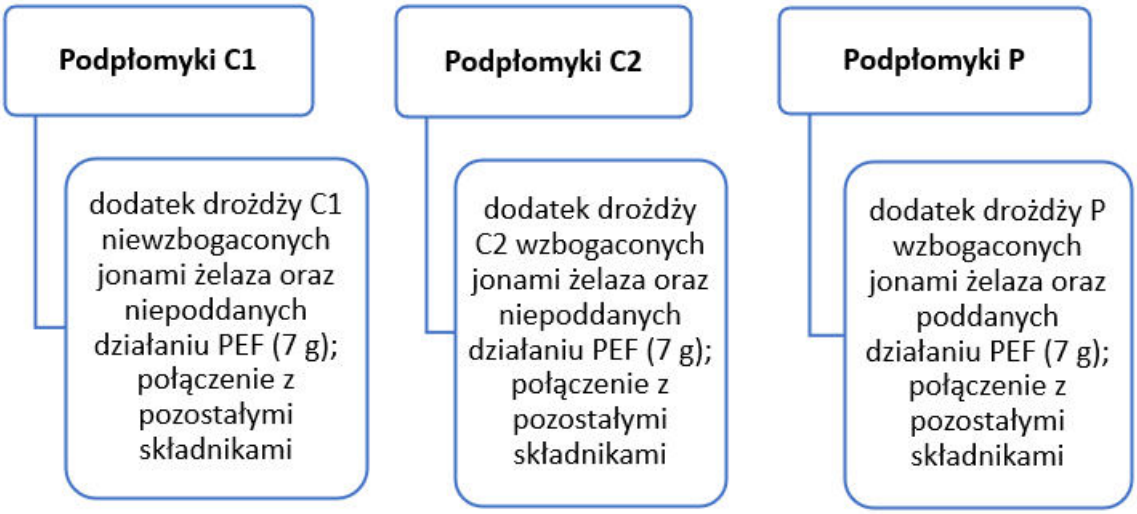
Rysunek 3. Schematyczne przedstawienie pojedynczej elektrody (A) i zestawu elektrod zanurzonych w pożywce hodowlanej (B).

2. Sterylizację pożywki przeprowadzono w autoklawie przy następujących parametrach: temperatura 121°C, ciśnienie 0,05 MPa, czas 20 minut.

3. Zawartość jonów żelaza w komórkach drożdży oraz produktach oznaczano metodą atomowej spektrometrii absorpcyjnej (FAAS). Próby uprzednio poddawano mineralizacji w piecu mikrofalowym MARS (CEM Corporation). Zawartość witaminy B_{12} w komórkach drożdży oraz produktach oznaczono metodą wysokosprawnej chromatografii cieczowej (HPLC).
4. Biomasę drożdży oznaczono spektrofotometrycznie w aparacie Spekol 11 (Carl Zeiss, Jena, Germany). Absorbancję pozorną wyznaczono przy długości fali α=600 nm i drodze optycznej równej 2 mm.
5. Oznaczono liczbę martwych komórek drożdży zabarwionych 0,01% roztworem błękitu metylenowego w komorze Thoma. Procent komórek martwych wyrażono jako średnią z 16 pól obliczonych według wzoru:

$$\%\ komórek\ martwych = (liczba\ komórek\ martwych)/(suma\ komórek\ martwych\ i\ żywych)\times 100\ \%$$

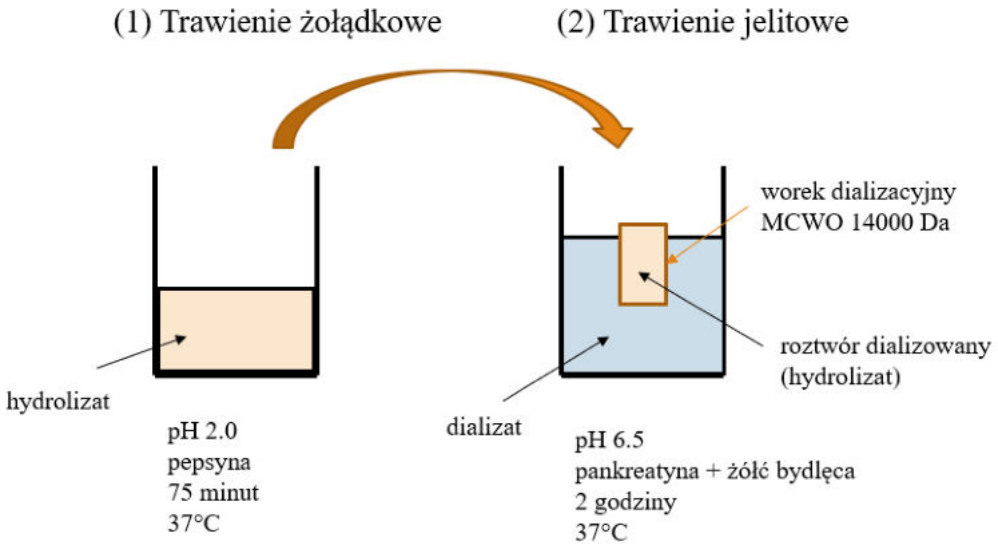
6. Aktywność fermentacyjną drożdży oznaczono przez pomiar zmian objętości ciasta rosnącego w czasie 0-120 min.
7. Próbki obserwowano pod mikroskopem fluorescencyjnym (Eclipse 90i, Nikon, Tokio, Japonia). Długość fali wzbudzenia i emisji wynosiła odpowiednio 550 nm i 580 nm.
8. Widma absorpcyjne w średniej podczerwieni uzyskano za pomocą spektroskopii osłabionego całkowitego odbicia w podczerwieni (ATR-FTIR) IRSpirit (Shimadzu, Kioto, Japonia) wyposażonej w detektor DLATGS. Pomiary przeprowadzono przy użyciu akcesorium ATR QATR™-S Single-Reflection z diamentowym kryształem (Shimadzu).
9. Podpłomyki wyprodukowano według schematu na rysunku 4. Poza drożdżami użyto do ich przygotowania 450 g mąki pszennej (biała mąka pszenna do ciast, typ 450), sól, oliwę z oliwek i 30 ml ciepłej wody.



Rysunek 4. Schemat produkcji podpłomyków zawierających drożdże wzbogacone w jony żelaza za pomocą PEF

10. Płatki drożdżowe (próbki C1, C2, P) przygotowano z dodatkiem drożdży niewzbogaconych jonami żelaza i witaminy B_{12} (Sigma-Aldrich, St. Louis, MO, USA) (Y1), drożdży wzbogaconych jonami żelaza oraz witaminą B_{12} tylko poprzez uzupełnienie pożywki (Y3) oraz drożdży wzbogaconych jonami żelaza i witaminą B_{12} poprzez uzupełnienie pożywki i zastosowanie PEF (Y5).
11. Oznaczono podstawowe składniki odżywcze:
    - tłuszcz w produktach wykorzystując metodę ekstrakcji w aparacie Soxhleta (Tecator Soxtec System HT 1043 extractie unit, Apeldoorn);
    - zawartość białka w produktach metodą Kiejdahla (AOAC, 2010);
    - popiół zgodnie z normą PN-EN ISO 2171:2010;
    - zawartość węglowodanów obliczono jako różnicę między 100% a sumą procentowej zawartości wszystkich pozostałych składników (wody, białka, tłuszczu, popiołu).
    - suchą masę oznaczono metodą suszarkową (130±1°C przez 3 godziny).
12. Analizę barwy wykonano stosując kolorymetr EnviSense NH310 (EnviSense, Lublin, Polska) w skali CIE – L*a*b*. Wyznaczone średnie parametrów barwy (3 powtórzenia) posłużyły do wyznaczenia całkowitej różnicy barwy (ΔE).
13. Potencjał antyoksydacyjny oznaczono spektrofotometrycznie poprzez ocenę zdolności do neutralizowania wolnych rodników generowanych z ABTS i DPPH.

14. Indeks glikemiczny (IG) podpłomyków i płatków drożdżowych określono metodą Reisa i Abu-Ghannama (2014) z niewielkimi modyfikacjami opisanymi w **PIII i PIV**.
15. Trawienie in vitro (rys. 5) przeprowadzono według Szalast-Pietrzak i in. (2018) z niewielkimi modyfikacjami opisanymi w **PIII i PIV**.



Rysunek 5. Schemat trawienia in vitro: (1) trawienie w żołądku: enzym: pepsyna, pH: 2,0, czas trawienia: 75 minut w temperaturze 37°C; (2) trawienie jelitowe: enzymy: pankreatyna i żółć, pH: 6,5, czas trawienia: 2 godziny w temperaturze 37°C.

16. Ocenę sensoryczną podpłomyków i płatków drożdżowych przeprowadzono metodą 5-punktową.
17. Analizy regresji i testy istotności przeprowadzono przy użyciu oprogramowania Statistica w wersji 13.3 (StatSoft, Inc., Tulsa, OK, USA). Do określenia różnic między średnimi zastosowano test post-hoc Tukeya. Wyniki $p<0,05$ uznano za istotne statystycznie. Wszystkie oznaczenia wykonano w 3 powtórzeniach.

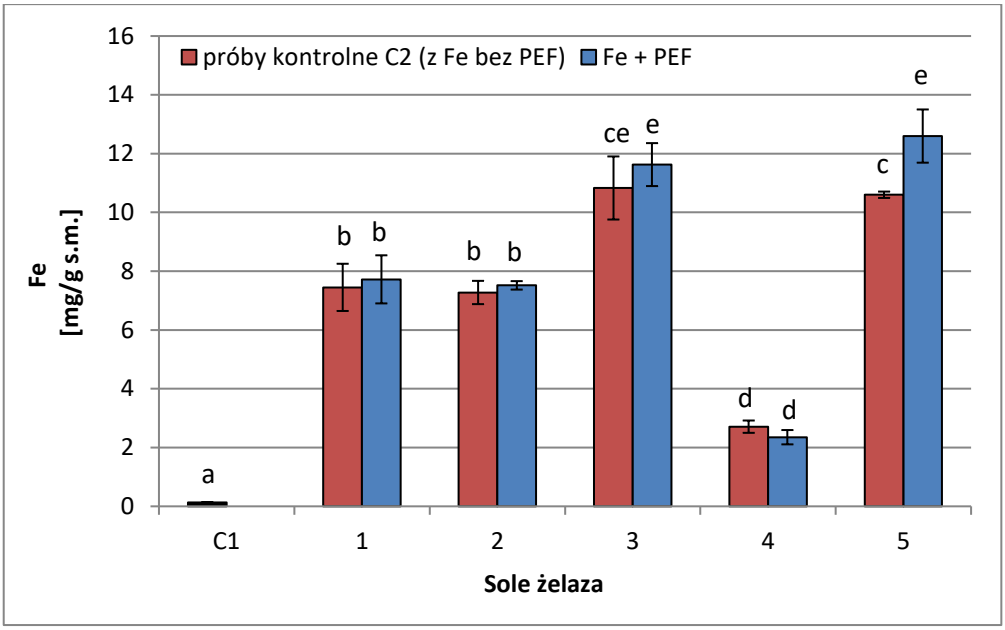
Metodykę szczegółowo opisano w poszczególnych publikacjach wchodzących w skład rozprawy doktorskiej (zgodnie z wykazem w rozdziale 1).

5. Prezentacja wybranych wyników badań

5.1. Wybór soli żelaza oraz optymalnego stężenia jonów żelaza

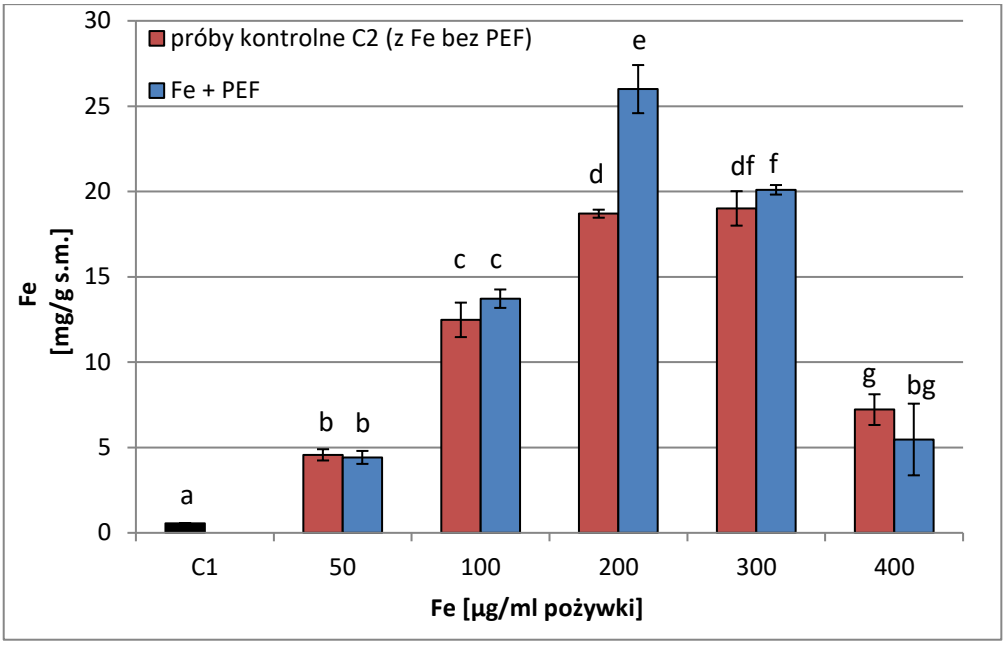
Pierwsza opublikowana praca (**PI**) wchodząca w skład dysertacji jest przeglądem piśmiennictwa opisującym mechanizm działania pulsacyjnego pola elektrycznego na komórki drożdży oraz bakterii oraz podsumowującym zastosowanie PEF w technologii żywności i w produkcji żywności potencjalnie funkcjonalnej.

Celem publikacji **PII** było zbadanie wpływu pulsacyjnego pola elektrycznego na akumulację jonów żelaza w komórkach *S. cerevisiae.* W pierwszym etapie doświadczenia wyselekcjonowano sól żelaza, dla której zanotowano największą akumulację tego pierwiastka w drożdżach. Komórki pochodzące z hodowli bez dodatku soli żelaza i bez PEF zawierały jedynie 0,13 mg Fe/g suchej masy i stanowiły kontrolę C1 (rys. 6). Badania wykazały, że pobieranie jonów żelaza z pożywki zachodziło najefektywniej gdy ich źródłem były chlorek żelaza (III) oraz azotan (V) żelaza (III). Może to być związane z faktem, że są to sole mocnego kwasu i prawdopodobnie występują w postaci zdysocjowanej, dostarczając tym samym wolne żelazo do wiązania się z komórkami (Philpott i Protchenko, 2008). Zastosowanie PEF o wyjściowych parametrach zwiększyło akumulację jonów żelaza z tych soli, ale wzrost ten był statystycznie istotny tylko dla azotanu (V) żelaza (III). W przypadku tej soli stężenie jonów żelaza w komórkach drożdży było prawie 97 razy wyższe w próbce traktowanej PEF niż w próbce kontrolnej bez dodatku soli żelaza i niepoddanej PEF (C1). Drożdże akumulowały najmniejszą ilość jonów żelaza z cytrynianu żelaza (III), co może być spowodowane niepełną dysocjacją tego związku w pożywce o pH 4,4 (Nielsen i Arneborg, 2007). Dodatkowo niektórzy autorzy zaobserwowali toksyczne działanie cytrynianu żelaza (III) na komórki drożdży (Paš i in., 2007; Chen i in., 2002).



Rysunek 6. Wpływ soli żelaza na akumulację żelaza w komórkach drożdży: C1 — hodowla kontrolna bez dodanych do pożywki jonów żelaza i PEF; 1 – $FeCl_2x4H_20$; 2 – $FeSO_4x6H_2O$; 3 – $FeCl_3x6H_2O$; 4 – $C_6H_5FeO_7$; 5 – $Fe(NO_3)_3x9H_2O$. Czerwone słupki - hodowle nietraktowane PEF, niebieskie słupki - hodowle traktowane PEF (100 µg Fe/ml pożywki, napięcie 1500 V, szerokość impulsu 10 µs, czas działania PEF 10 min, liczba impulsów 600, po 20 h hodowli). Każda wartość jest średnią ± odchylenie standardowe (n = 3). Słupki z tą samą literą (a–e) nie różnią się istotnie (p<0,05).

W kolejnych etapach badań optymalizowano stężenie jonów żelaza w pożywce. Wykazano, że w zakresie stężenia 50–100 µg Fe^{3+}/ml pożywki działanie PEF nie miało wpływu na akumulację żelaza w drożdżach. Statystycznie istotne zmiany odnotowano przy 200 µg Fe^{3+}/ml pożywki, a różnica w akumulacji między komórkami suplementowanymi solą żelaza bez traktowania PEF (C2), a komórkami traktowanymi PEF (P) była największa. Zaobserwowano, że pobieranie jonów żelaza zmniejszało się, gdy stężenie było wyższe niż 200 µg Fe^{3+}/ml pożywki (rys. 7), więc przyjęto stężenie 200 µg Fe^{3+}/ml pożywki jako optymalne dla efektywnej akumulacji tego pierwiastka w dalszych doświadczeniach.



Rysunek 7. Wpływ stężenia żelaza na jego akumulację w komórkach drożdży. C1 - hodowla kontrolna bez jonów żelaza dodanych do pożywki i obróbki PEF; czerwone słupki - hodowle niepoddane działaniu PEF, niebieskie słupki - hodowle poddane działaniu PEF (azotan (V) żelaza (III), napięcie 1500 V, szerokość impulsu 10 µs, czas działania PEF 10 min, liczba impulsów 600, po 20 h hodowli). Każda wartość jest średnią ± odchylenie standardowe (n = 3). Słupki z tą samą literą (a–g) nie różnią się istotnie (p<0,05).

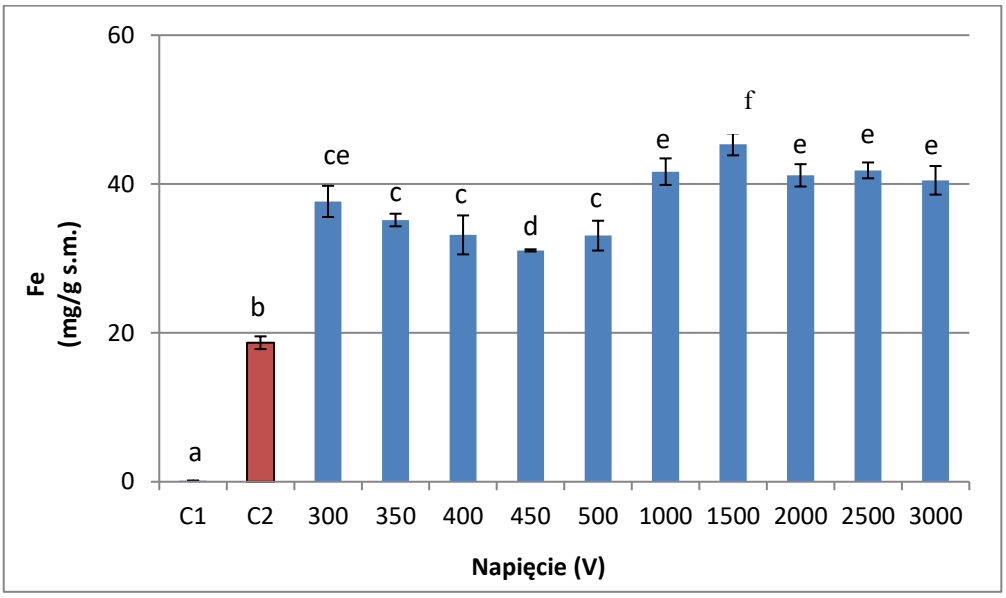
Żelazo jest niezbędne do wzrostu drożdży, ale jego wysoka zawartość w pożywce może być również toksyczna. Z tego powodu pobieranie i wykorzystanie żelaza w komórkach drożdży jest ściśle regulowane (Martínez-Pastor i in., 2017). Badania Philpota i Protchenko (2008) wskazują, że gdy ilość żelaza jest ograniczona, komórki nie tylko zwiększają pobieranie tego pierwiastka, ale także dostosowują swój metabolizm, aby wydajniej wykorzystywać dostępne żelazo.

5.2. Optymalizacja parametrów PEF i jej wpływ na akumulację jonów żelaza w komórkach drożdży

Rysunki 8, 9 i 10 przedstawiają wpływ parametrów PEF na akumulację żelaza w komórkach *Saccharomyces cerevisiae*.

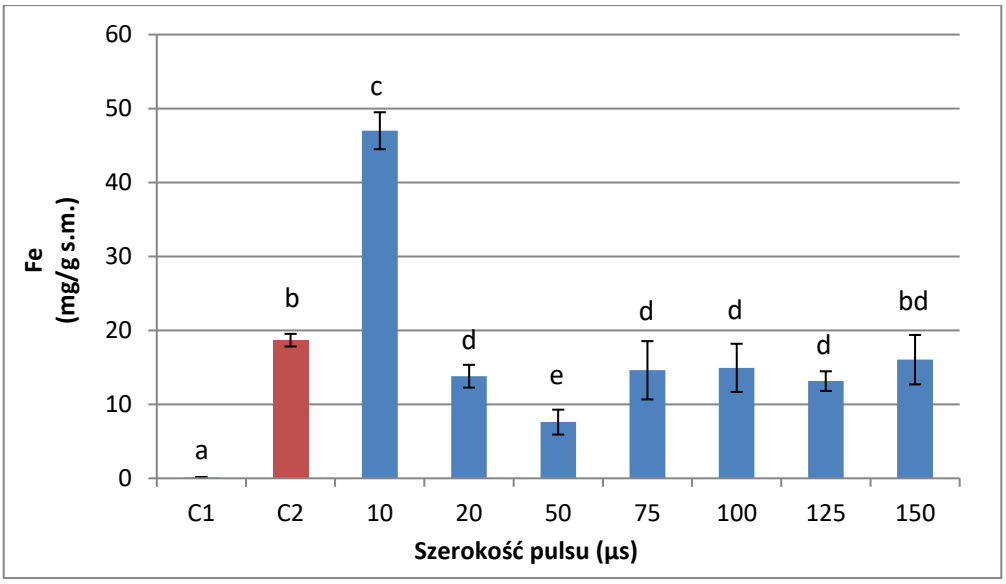
Zastosowanie niskich wartości napięcia PEF (300–500 V) spowodowało dwukrotny wzrost zawartości żelaza w komórkach drożdży w porównaniu z próbką kontrolną C2 (rys. 8). Największą akumulację jonów żelaza (ponad 2,6 razy wyższą niż w C2) osiągnięto przy

napięciu wynoszącym 1500 V. Wyższe wartości napięcia powodowały istotne obniżenie zawartości jonów żelaza w komórkach drożdży.



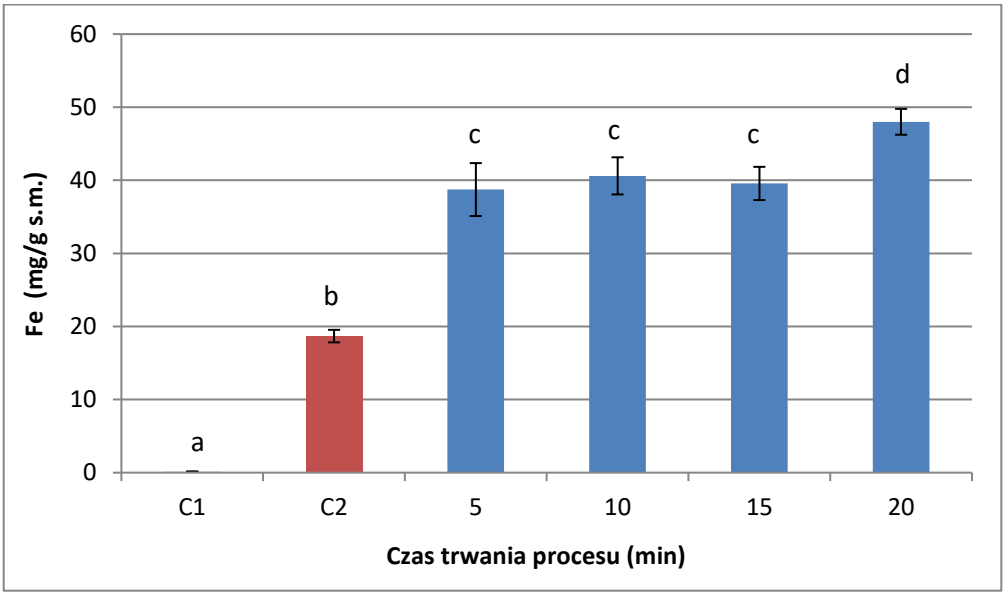
Rysunek 8. Wpływ napięcia PEF na akumulację żelaza w komórkach drożdży. C1 — hodowla kontrolna bez jonów żelaza dodanych do pożywki i PEF, C2 — hodowla kontrolna z jonami żelaza dodanymi do pożywki (200 µg Fe^{3+}/ml pożywki) i bez PEF, niebieskie słupki — hodowle poddane działaniu PEF (azotan (V) żelaza (III), 200 µg $Fe^{3+/}$ml pożywki, szerokość impulsu 10 µs, czas działania PEF 10 min, liczba impulsów 600, po 20 h hodowli). Każda wartość jest średnią ± odchylenie standardowe (n = 3). Słupki z tą samą literą (a–f) nie różnią się istotnie (p<0,05).

Najwyższe stężenie jonów żelaza w komórkach tj. 47 mg Fe^{3+}/g s.m, odnotowano przy szerokości pulsu 10 µs (rys. 9). Stężenie jonów żelaza w komórkach poddanych działaniu PEF przy 20, 50 i 75 µs, a także przy 100, 125 i 150 µs nie różniło się istotnie.



Rysunek 9. Wpływ szerokości pulsu na akumulację żelaza w komórkach drożdży. C1 - hodowla kontrolna bez jonów żelaza dodanych do pożywki i PEF, C2 - hodowla kontrolna z jonami żelaza dodanymi do pożywki (200 µg Fe^{3+}/ml pożywki) i bez PEF, niebieskie słupki - kultury poddane działaniu PEF (azotan żelaza, 200 µg Fe^{3+}/ml pożywki, napięcie 1500 V, czas działania PEF 10 min, ilość impulsów 600, po 20 h hodowli). Każda wartość jest średnią ± odchylenie standardowe (n = 3). Słupki z tą samą literą (a–d) nie różnią się istotnie (p<0,05).

Badania nad czasem działania PEF prowadzono w zakresie 5–20 min (rys. 10). Stężenie jonów żelaza w komórkach *S. cerevisiae* wzrastało wraz z czasem, osiągając maksimum (48,01 mg Fe^{3+}/g suchej masy) po 20 minutach.



Rysunek 10. Wpływ czasu działania PEF na akumulację jonów żelaza w komórkach drożdży. C1 - hodowla kontrolna bez jonów żelaza dodanych do pożywki i PEF, C2 - hodowla kontrolna z jonami

żelaza dodanymi do pożywki (200 μg Fe^{3+}/ml pożywki) i bez PEF, niebieskie słupki - hodowle poddane działaniu PEF (azotan (V) żelaza (III), 200 μg Fe^{3+}/ml pożywki, napięcie 1500 V, szerokość impulsu 10 μs, po 20 h hodowli). Każda wartość jest średnią ± odchylenie standardowe (n = 3). Słupki z tą samą literą (a–d) nie różnią się istotnie (p<0,05).

Temperatura jest jednym z krytycznych parametrów wpływających na skuteczność pulsacyjnego pola elektrycznego (Raso i in., 2016). W prowadzonych eksperymentach temperatura była stale monitorowana i wynosiła od 24 do 26°C. Nie było drastycznych zmian, które mogłyby wpłynąć na przewodnictwo pożywki lub wzrost komórek.

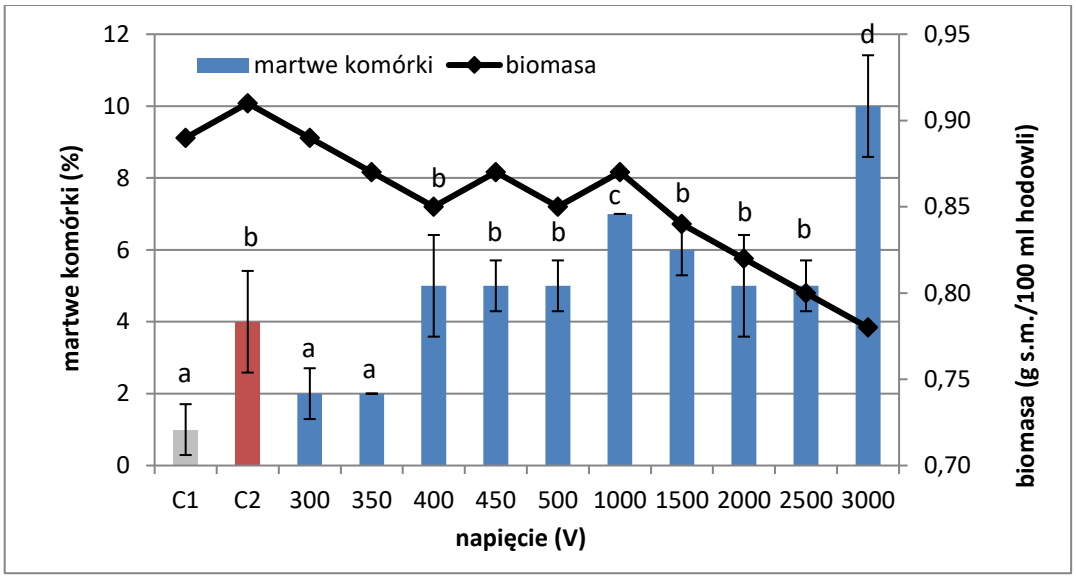
Pankiewicz i Jamroz (2011) również zaobserwowali najwyższą akumulację cynku w komórkach *S. cerevisiae* przy szerokości pulsu 10 μs (15 mg/g suchej masy). W przypadku tych badań optymalny czas działania PEF wynosił 15 minut. Różnica w czasie trwania procesu między badaniami przedstawionymi w rozprawie doktorskiej a badaniami Pankiewicz i Jamroza (2011) może być spowodowana wbudowaniem innego pierwiastka do komórek *S. cerevisiae.*

W badaniach Pankiewicz i in. (2014, 2015) optymalny czas hodowli, po którym komórki drożdży zostały poddane PEF w celu wzbogacenia w magnez i cynk oraz selen również wynosił 20 godzin. W dostępnych w literaturze badaniach nad akumulacją jonów żelaza z pożywki przez drożdże występują jednak znaczne rozbieżności w optymalnym czasie hodowli, co może wynikać z różnych warunków jej prowadzenia. Na przykład Stehlik-Thomas i in. (2003) stwierdzili, że najwyższe stężenie żelaza w komórkach (10 mg/g suchej biomasy drożdży) uzyskano po 12 h hodowli w warunkach beztlenowych. Natomiast w warunkach półtlenowych najwyższą akumulację osiągnięto po 16 h hodowli, ale była ona czterokrotnie niższa (2,5 mg/g suchej biomasy drożdży) niż ww. Wang i in. (2011) z kolei najwyższą zawartość Fe (7,854 mg/g suchej masy) uzyskali w drożdżach hodowanych przez 60 h w temperaturze 30°C.

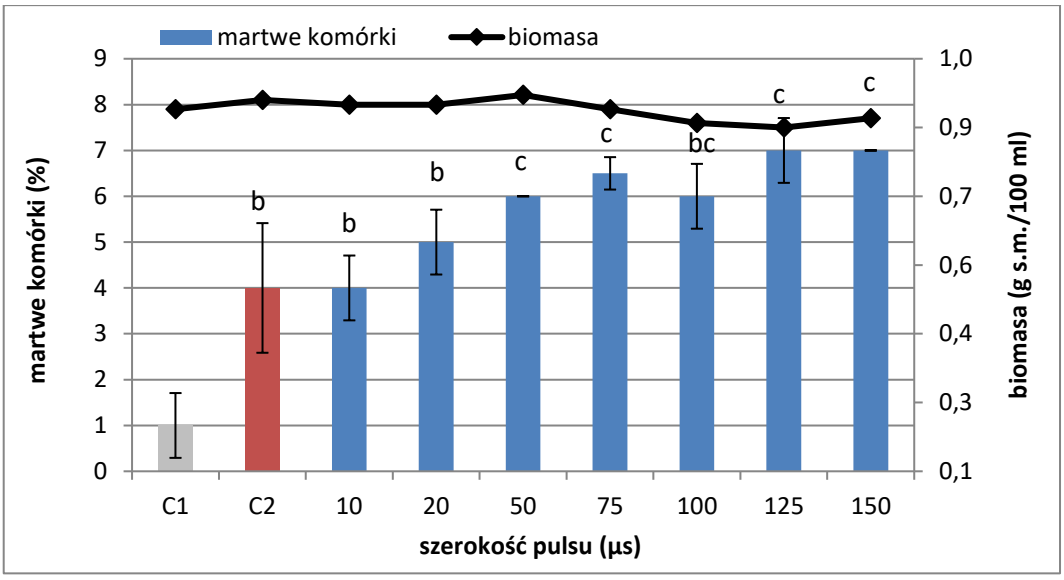
5.3. Ocena zmian biomasy i przeżywalności drożdży po działaniu PEF

Przedstawione w publikacji **PII** wyniki oznaczania biomasy oraz przeżywalności drożdży były wysokie. Rysunki 11, 12 oraz 13 przedstawiają wpływ parametrów PEF na produkcję biomasy i żywotność komórek. Zaobserwowano jedynie niewielkie wahania liczby martwych komórek w całym zakresie badanych wartości napięcia. Największą frakcję martwych komórek w hodowli (10%) zanotowano przy 3000 V (rys. 11). Istotny spadek produkcji biomasy (z 0,87 do 0,78 g s.m./100 ml hodowli) odnotowano tylko przy napięciach

wyższych niż 2000 V. W przypadku optymalizacji szerokości pulsu, istotny spadek biomasy nastąpił w zakresie od 75 µs do 150 µs przy 1500 V (rys. 12). Szerokość pulsu wpłynęła również na żywotność komórek drożdży. Przy 10 µs był taka sama jak w kontroli niepoddanej działaniu PEF (C2), jednak dla wartości wyższych niż 50 µs żywotność komórek uległa niewielkiemu obniżeniu. Mimo to nadal notowano wysokie wartości dla obu parametrów – żywotność przekraczała 90%, a produkcja biomasy wynosiła ok. 0,95 g s.m./100 ml hodowli.

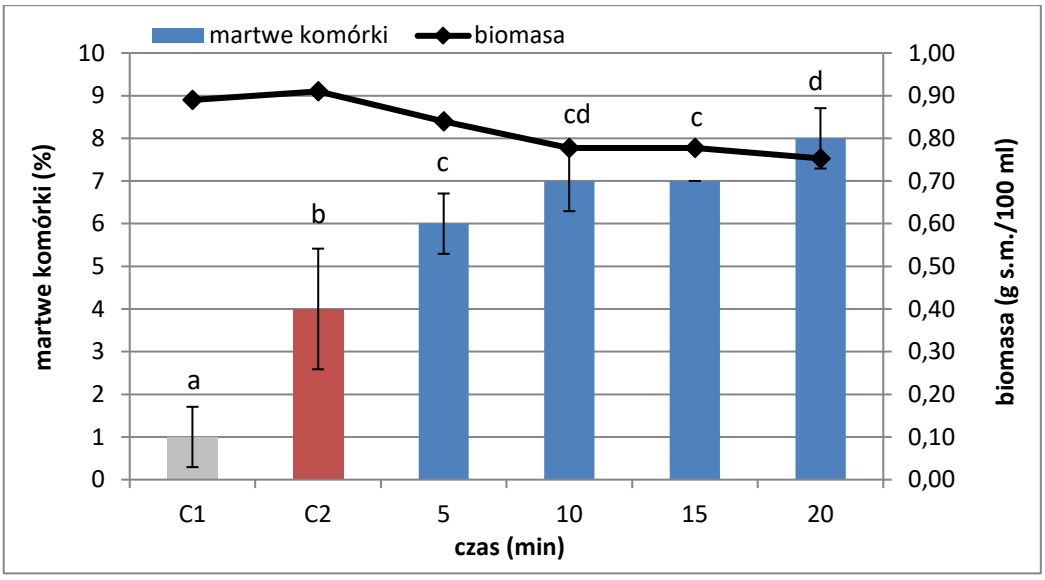


Rysunek 11. Wpływ napięcia PEF na żywotność komórek i biomasy. C1 - hodowla kontrolna bez jonów żelaza dodanych do pożywki i PEF, C2 - hodowla kontrolna z jonami żelaza dodanymi do pożywki (200 µg Fe^{3+}/ml pożywki) i bez PEF, niebieskie słupki - hodowle poddane działaniu PEF (azotan (V) żelaza (III), 200 µg Fe^{3+}/ml pożywki, szerokość impulsu 10 µs, czas działania PEF 10 min, liczba impulsów 600, po 20 h hodowli). Każda wartość jest średnią ± odchylenie standardowe (n=3). Słupki z tą samą literą (a–c) nie różnią się istotnie (p<0,05).



Rysunek 12. Wpływ szerokości pulsu na żywotność komórek i biomasy. C1 - hodowla kontrolna bez jonów żelaza dodanych do pożywki i PEF, C2 - hodowla kontrolna z jonami żelaza dodanymi do pożywki (200 µg Fe^{3+}/ml pożywki) i bez PEF, niebieskie słupki - hodowle poddane działaniu PEF (azotan (V) żelaza (III), 200 µg Fe^{3+}/ml pożywki, napięcie 1500 V, czas działania PEF 10 min, ilość impulsów 600, po 20 h hodowli). Szerokość impulsu zmieniano od 10 µs do 150 µs przy amplitudzie napięcia 1500 V. Każda wartość jest średnią ± odchylenie standardowe (n=3). Słupki oznaczone samą literą (a–c) nie różnią się istotnie (p<0,05).

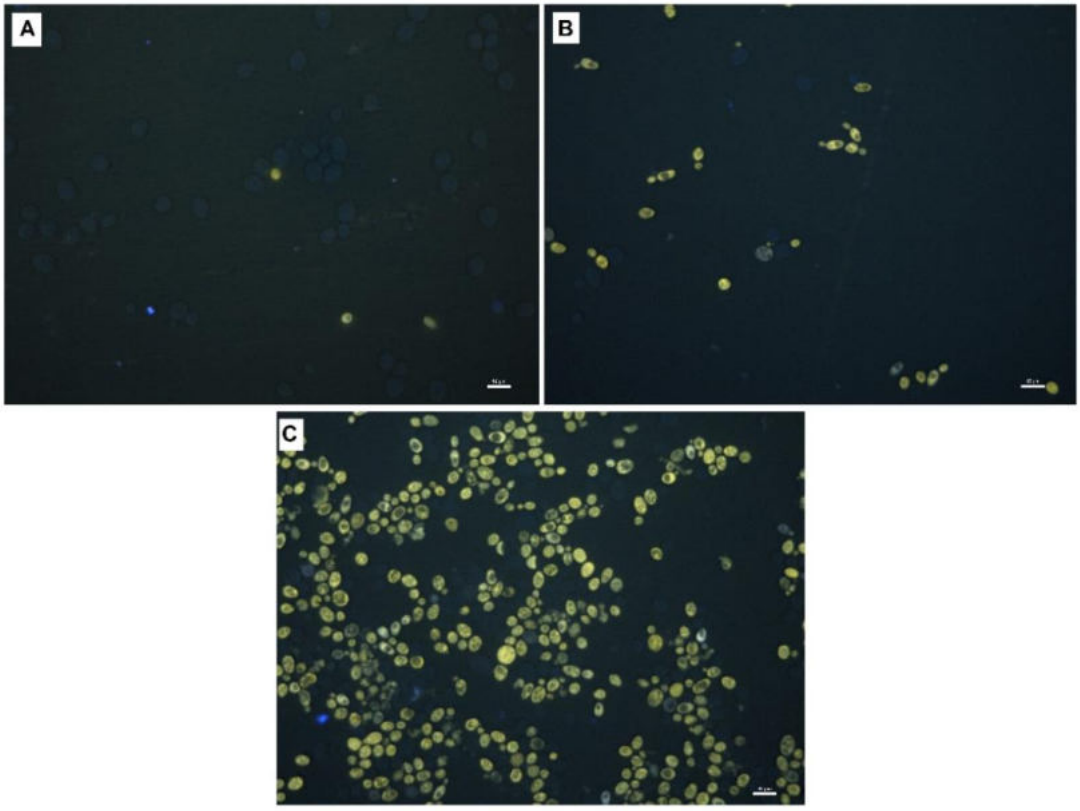
Czas działania PEF miał niewielki wpływ na biomasę i żywotność komórek (rys. 13). Chociaż produkcja biomasy obniżyła się już po 5 minutach elektroporacji, w porównaniu z hodowlami kontrolnymi, spadek ten nie był znaczący. Po 20 minutach działania PEF produkcja biomasy była o 15% niższa niż w hodowli kontrolnej C2, ale całkowita liczba martwych komórek była niska (tylko 8%). Dłuższe działanie PEF skutkowało większym spadkiem produkcji biomasy i żywotności komórek.



Rysunek 13. Wpływ działania PEF na żywotność komórek i biomasy. C1 - hodowla kontrolna bez jonów żelaza dodanych do pożywki i PEF, C2 - hodowla kontrolna z jonami żelaza dodanymi do pożywki (200 µg Fe^{3+}/ml pożywki) i bez PEF, niebieskie słupki - hodowle poddane działaniu PEF (azotan (V) żelaza (III), 200 µg Fe^{3+}/ml pożywki, napięcie 1500 V, szerokość impulsu 10 µs, po 20 h hodowli). Każda wartość jest średnią ± odchylenie standardowe (n=3). Słupki z tą samą literą (a–b) nie różnią się istotnie (p<0,05).

5.4. Ocena wpływu PEF na akumulację jonów żelaza w strukturach komórkowych drożdży metodą mikroskopii fluorescencyjnej oraz ATR-FTIR

Rysunek 14 przedstawia komórki drożdży wybarwione rodaminą B i obserwowane pod mikroskopem fluorescencyjnym. Barwnik ten przenika przez błony komórkowe i jest wychwytywany przez mitochondria bez wywoływania lizy komórek. Prawie wszystkie komórki z próbki kontrolnej niepoddanej PEF i bez suplementacji solą żelaza nie wykazywały fluorescencji (rys. 14A), podczas gdy te z próbki wzbogaconej jonami żelaza bez PEF częściowo wykazywały zieloną fluorescencję (rys. 14B). Działanie PEF zwiększyło akumulację jonów żelaza w komórkach drożdży, więc prawie cała populacja komórek wykazała silną zieloną fluorescencję (rys. 14C).



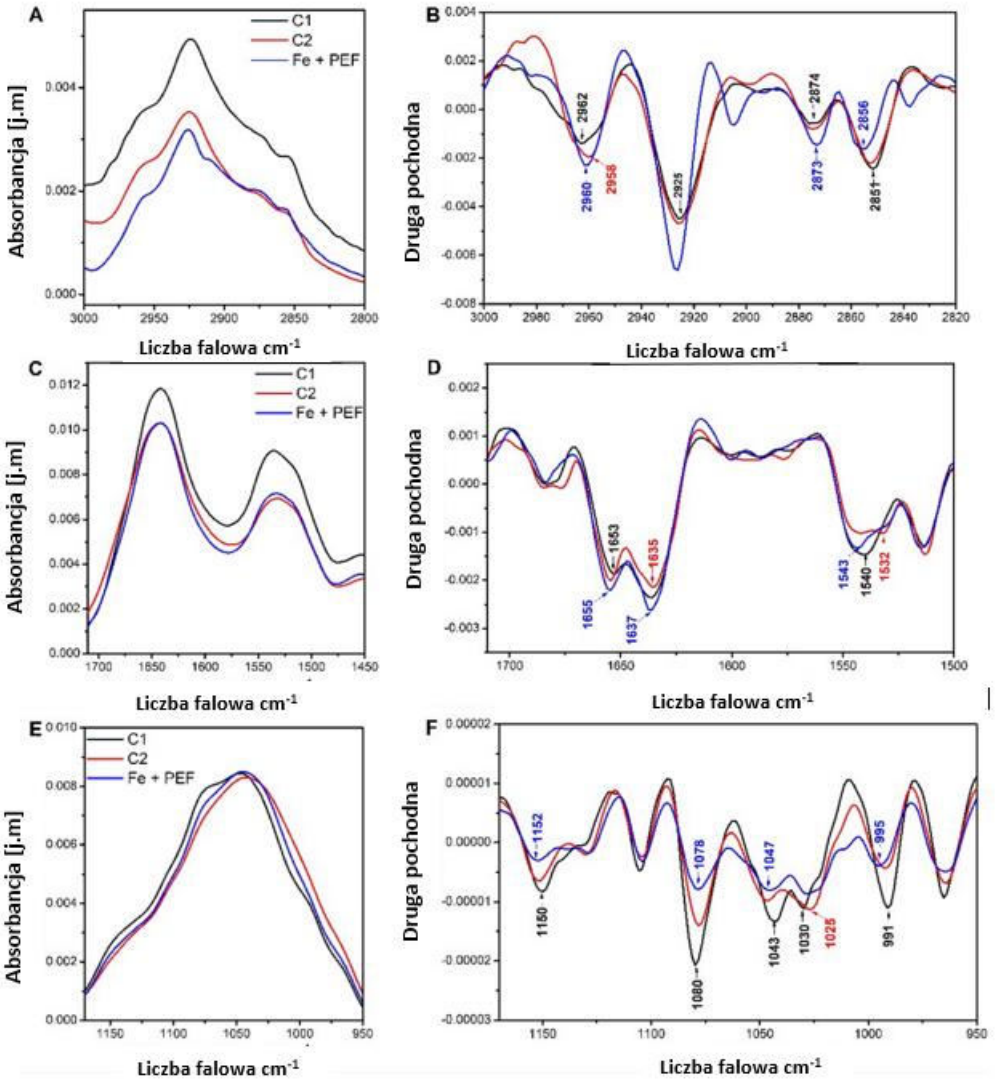
Rysunek 14. Obrazy mikroskopii fluorescencyjnej drożdży barwionych rodaminą B: (A) C1 - hodowla kontrolna bez jonów żelaza dodanych do pożywki i traktowania PEF, (B) C2 - hodowla kontrolna z jonami żelaza dodanymi do pożywki (200 μg Fe^{3+}/ml pożywki) i bez PEF, (C) hodowla z dodanym żelazem do pożywki (200 μg $Fe3^{+}$/ml pożywki) i traktowane PEF (azotan (V) żelaza (III), napięcie 1500 V, szerokość impulsu 10 μs, czas działania PEF 20 min, 1200 impulsów, po 20 h hodowli). Słupki skali odpowiadają 10 μm.

Pankiewicz i in. (2015) prowadzili badania nad akumulacją jonów wapnia i cynku przez drożdże w tym samym systemie obróbki PEF, który zastosowano w przypadku badań przedstawionych w rozprawie doktorskiej. Ich badania wykazały, że fluorescencja komórek z próbek kontrolnych (bez jonów dodanych do podłoża i bez obróbki PEF) była niższa niż obserwowana dla komórek z próbki wzbogaconej w jony wapnia i cynku przy użyciu PEF. Na podstawie przekrojów optycznych wykonano również rekonstrukcje 3D rozmieszczenia obszarów bogatych w jony w komórce. Potwierdzono, że w komórkach drożdży poddanych elektoporacji akumulacja jonów wapnia i cynku była wyższa niż w tych nie poddanych działaniu PEF, a jony metali były rozmieszczone wewnątrz komórki.

Widma ATR-FTIR liofilizowanych komórek drożdży z próbki kontrolnej (C1), próbek wzbogaconych w jony żelaza zarówno bez obróbki PEF (C2), jak i z użyciem PEF w optymalnych warunkach (Fe + PEF) zarejestrowano w obszarze między 4000 i 750 cm^{-1}.

W widmach zaprezentowanych na rys. 15 zaobserwować można zmiany w obrębie regionu lipidowego (3000–2800 cm^{-1}), które są związane ze znacznym wzrostem intensywności pasm CH_2 przy 2925 cm^{-1} w przypadku próbek poddanych PEF (rys. 15B). Ponadto występuje również niewielki wzrost intensywności absorpcji CH_3 przy ~2960 i ~2873 cm^{-1} dla obu próbek z dodatkiem jonów żelaza. Ta zmienność udziału CH_2 i CH_3 może być związana z indukowaniem zmiany płynności błony drożdży przez jony żelaza. Ponadto zaobserwowano przesunięcie o 1–4 cm^{-1} w kierunku wyższych liczb falowych pasm rozciągających asymetrycznych i symetrycznych dla grupy CH_2, zwłaszcza u drożdży poddanych PEF (rys. 15B). Stan lipidowych błon komórkowych jest powiązany z przesunięciem pasm widmowym CH_2 w kierunku niższych lub wyższych częstotliwości i odpowiada odpowiednio ich sztywności lub płynności (Los i Murata, 2004). Ganeva i in. (2014) oraz Stirke i in. (2014) stwierdzili, że działanie na drożdże pulsacyjnym polem elektrycznym może skutkować nie tylko zwiększeniem jej przepuszczalności, ale także powodować zmiany w strukturze ściany komórkowej, prowadząc do zwiększenia jej porowatości. Kiedy porównuje się komórki drożdży z hodowli kontrolnej i hodowli wzbogaconych w jony żelaza, profile drugiej pochodnej amidu I i II są dość podobne, z wyjątkiem położenia pasm (rys. 15D). Pozycja piku przy 1653 cm^{-1} (przypisana do helisy α) i 1635 cm^{-1} (przypisana do β-kartki i najprawdopodobniej wody wewnątrzkomórkowej) dla próbek C2 oraz Fe+PEF uległa zmianie w porównaniu z próbką C1. To przesunięcie pasma amidowego I do 1655 cm^{-1} było związane z zaangażowaniem atomów O i N łańcucha polipeptydowego w wiązanie jonów żelaza (rys. 15D). Zmiany kształtu szerokiego piku w zakresie 1180–950 cm^{-1} dla drożdży wzbogaconych w jony żelaza w porównaniu z próbką kontrolną C1 mogą wskazywać na interakcje jonów żelaza z polisacharydami obecnymi w ścianie komórkowej. Większość polisacharydów komórki drożdży znajduje się w jej ścianie (Nguyen i in., 1998). Dokładniej, wewnętrzna warstwa ściany składa się głównie z β-1,3-glukanu, ale zewnętrzna jej warstwa jest utworzona przez wysoce glikozylowane mannoproteiny z licznymi grupami fosforanowymi w ich węglowodanowych łańcuchach bocznych, co skutkuje ujemnym ładunkiem powierzchniowym. W drugiej pochodnej widma drożdży po działaniu PEF, pasmo przypisywane β-1,3-glukanowi (1150 cm^{-1}) jest znacznie mniej intensywne, poszerzone i przesunięte w kierunku wyższej częstotliwości. Biorąc pod uwagę inne pasma związane z węglowodanami, zaobserwowano również spadek intensywności pasm przypisanych do struktury glukanu, a mianowicie przy 1080 cm^{-1}, 1043 cm^{-1} (mannany), 1030 i 991 cm^{-1} (β-1,6 glukanów) i ich przesunięcie w porównaniu widmem dla kontroli C1 (rys. 15F). Wreszcie, silna redukcja pasma przy

około 1080 cm^{-1} obserwowana w próbce drożdży po działaniu PEF może sugerować interakcję grupy PO_2- z fosfolipidów błonowych z dodatnimi jonami żelaza (Zinicovscaia i in., 2020). Dzięki temu jony żelaza mogły przejść przez ścianę komórkową i przestrzeń peryplazmatyczną i dotrzeć na powierzchnię błony plazmatycznej, w dużym stopniu w wyniku działania PEF.

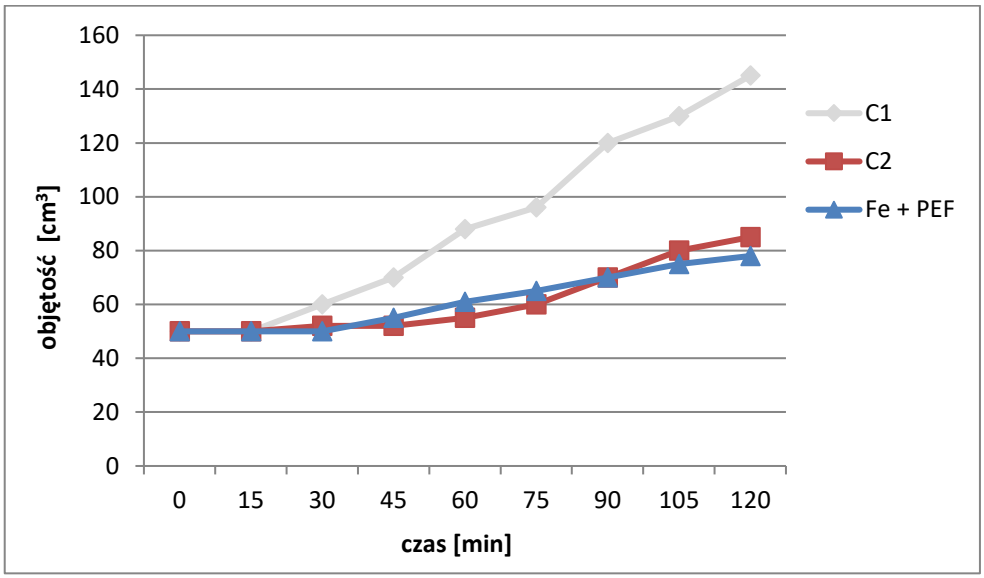


Rysunek 15. Widma ATR FTIR drożdży i ich drugie pochodne w wybranych zakresach. (A,B): obszar modów wibracyjnych spowodowanych głównie drganiami rozciągającymi asymetrycznymi i symetrycznymi grup metylenowych CH_2 i CH_3 (C,D): obszar pasm amidu I i amidu II; (E, F): region modów węglowodanów. Widma drugiej pochodnej zostały znormalizowane do pasma CH_2 przy ~2924 cm^{-1} (B), do amidu I (D), podczas gdy w panelu widma F zostały znormalizowane do obszaru w zakresie 1180-900 cm^{-1}. Hodowla kontrolna C1 bez dodatku żelaza do pożywki i traktowania PEF (czarna linia), hodowla kontrolna C2 z jonami żelaza dodanymi do pożywki (200 µg Fe^{3+}/ml pożywki) i bez traktowania PEF (linia czerwona) oraz hodowla Fe + PEF z jonami żelaza dodanymi do pożywki (200 µg Fe^{3+}/ml pożywki) i traktowana PEF (azotan (V) żelaza (III), napięcie 1500 V,

szerokość impulsu 10 µs, czas działania PEF 20 min, 1200 impulsów, po 20 h hodowli) (niebieska linia).

5.5. Analiza właściwości fermentacyjnych drożdży wzbogaconych w jony żelaza

Rysunek 16 przedstawia wyniki analizy właściwości fermentacyjnych drożdży uzyskane dla kontroli C1 i C2 oraz dla próbki drożdży wzbogaconej w jony żelaza w warunkach PEF (Fe + PEF). Kontrola C1 wykazała najwyższą aktywność fermentacyjną. Już w 30 minucie testu zaobserwowano wzrost ciasta, natomiast ciasto z dodatkiem drożdży z C2 i Fe + PEF zaczęło rosnąć dopiero po 60 min. Po 120 min objętość ciasta zawierającego drożdże z kontroli C1 była 1,7-krotnie większa niż ciasta z drożdżami z kontroli C2 i 1,85-krotnie większa niż w przypadku ciasta z drożdżami poddanymi działaniu PEF.



Rysunek 16. Wpływ PEF i wzbogacania w żelazo na właściwości fermentacyjne drożdży: C1 - hodowla kontrolna bez dodatku żelaza do pożywki i PEF, C2 - hodowla kontrolna z dodanymi jonami żelaza do pożywki (200 µg Fe^{3+}/ml pożywki) i bez PEF, Fe + PEF - hodowla z jonami żelaza dodanymi do pożywki (200 µg $Fe3^{+}$/ml pożywki) i traktowana PEF (azotan (V) żelaza (III), napięcie 1500 V, szerokość impulsu 10 µs, czas działania PEF 20 min, 1200 impulsów, po 20 h hodowli). Każda wartość jest średnią ± odchylenie standardowe (n=3).

Niższa wydajność fermentacyjna drożdży z próbki C2 i tych poddanych działaniu PEF może być również związana z obecnością wysokiego stężenia jonów żelaza w komórkach. Żelazo, obok potasu, magnezu, wapnia, manganu, miedzi i cynku, jest jednym z najważniejszych metali wpływających na procesy fermentacji drożdży (Walker,

2004). Nadmiar żelaza może być szkodliwy dla komórek, ponieważ pewne formy mogą być zaangażowane w reakcje redoks Fentona, które przyspieszają tworzenie reaktywnych form tlenu (ROS), takich jak rodniki hydroksylowe, które uszkadzają komórki na poziomie błon, białek i kwasów nukleinowych (Martínez-Garay i in., 2016).

Obniżenie aktywności fermentacyjnej próbki kontrolnej C2 i próbki poddanej działaniu PEF (Fe + PEF) w porównaniu z C1 można także częściowo tłumaczyć niższą zawartością białka (Tabela 1). Kolejnym czynnikiem wpływającym na fermentację drożdży jest dostępność przyswajalnego azotu. Drożdże *S. cerevisiae* nie są w stanie wykorzystać azotanu jako jedynego źródła azotu (Barnett i in., 1990), ponieważ pozbawione enzymów zależnych od molibdenu nie mogą go przyswoić (Zhang i in., 2011).

Tabela 1. Zawartość białka w drożdżach: C1 – bez dodatku jonów żelaza i bez PEF; C2 - z dodatkiem jonów żelaza (200 μg Fe^{3+}/ml pożywki) i bez PEF, Fe + PEF - z dodatkiem jonów żelaza (200 μg Fe^{3+}/ml pożywki) i poddanych działaniu PEF (azotan (V) żelaza (III), napięcie 1500 V, szerokość impulsu 10 μs, czas działania PEF 20 min, 1200 impulsów, po 20 h hodowli). Każda wartość jest średnią ± odchylenie standardowe (n=3). Średnie z tą samą literą (a–c) nie różnią się istotnie (p<0,05).

Próbka	Zawartość białka (%)	Zawartość żelaza (mg/g s.m.)
C1	$59,13 \pm 0,18^{a}$	$0,13 \pm 0,01^{a}$
C2	$58,24 \pm 0,35^{b}$	$18,68 \pm 0,86^{b}$
Fe + PEF	$54,07 \pm 0,11^{c}$	$48,01 \pm 0,88^{c}$

5.6. Wybrane właściwości podpłomyków z dodatkiem drożdży wzbogaconych jonami żelaza za pomocą PEF

W tabeli 2 (publikacja **PIII)** przedstawiono wartość odżywczą i indeks glikemiczny podpłomyków z dodatkiem drożdży niewzbogaconych i wzbogaconych jonami żelaza w warunkach działania PEF.

Wartość odżywcza podpłomyków zależała głównie od składu chemicznego mąki i innych składników użytych do jego przygotowania (Mir i in., 2014). Węglowodany stanowiły 58–62% składu odżywczego. Podpłomyki C1, C2 i P nie różniły się istotnie pod względem zawartości białka i tłuszczu, lecz zanotowano między nimi istotną różnicę w zawartości popiołu, co można tłumaczyć zwiększoną zawartością żelaza w próbkach wzbogaconych w jony tego pierwiastka. Ilość węglowodanów, tłuszczów i białek wpływa

na kaloryczność produktów. Stwierdzono statystycznie istotne różnice w wartości kalorycznej podpłomyków - największą wartość kaloryczną miały podpłomyki z dodatkiem drożdży kontrolnych C1, a najniższą - podpłomyki z dodatkiem drożdży wzbogaconych jonami żelaza przy użyciu PEF.

Tabela 2. Skład odżywczy, kaloryczność i indeks glikemiczny (GI) podpłomyków (zawartość podawana na suchą masę) otrzymanych z użyciem drożdży niewzbogaconych i wzbogaconych jonami żelaza

Podpłomyki	Białko (%)	Tłuszcz (%)	Węglowodany (%)	Popiół (%)	Wartość kaloryczna (kcal/100g)	Wartość kaloryczna (kJ/100g)	Indeks glikemiczny (IG)
C1	$11,85 \pm 0,42^{a}$	$4,50 \pm 0,33^{a}$	$62,09 \pm 0,48^{c}$	$21,56 \pm 0,19^{a}$	$336,23 \pm 2,38^{c}$	$1406,79 \pm 9,97^{c}$	$56,24 \pm 0,12^{a}$
C2	$11,44 \pm 0,14^{a}$	$4,81 \pm 0,18^{a}$	$59,42 \pm 0,18^{b}$	$24,33 \pm 0,33^{b}$	$326,70 \pm 2,18^{b}$	$1366,91 \pm 9,11^{b}$	$56,51 \pm 0,25^{a}$
P	$12,39 \pm 0,45^{a}$	$4,11 \pm 0,19^{a}$	$58,14 \pm 0,40^{a}$	$25,37 \pm 0,35^{c}$	$319,07 \pm 2,26^{a}$	$1334,97 \pm 9,45^{a}$	$56,23 \pm 1,34^{a}$

C1 - drożdże bez dodatku jonów żelaza i bez PEF; C2 - drożdże z dodatkiem jonów żelaza i bez PEF; P - drożdże z dodatkiem jonów żelaza i PEF. Każda wartość jest średnią ± odchylenie standardowe (n=3). Wyniki z tą samą literą w kolumnie nie różnią się istotnie (p<0,05).

W tabeli 3 przedstawiono zawartość żelaza i potencjalną biodostępność tego metalu z podpłomyków. Biodostępność definiuje się jako zdolność składnika odżywczego do uwolnienia się z matrycy żywności i rozpuszczenia. Decyduje o ilości substancji czynnej, która z podanej dawki przedostaje się do krążenia ogólnoustrojowego, a także o szybkości wchłaniania tej substancji (Moreda-Piñeiro i in., 2017).

Podpłomyki otrzymane z dodatkiem drożdży niewzbogaconych w żelazo zawierały tylko około 3 mg Fe^{3+}/100 g suchej masy, natomiast podpłomyki z dodatkiem drożdży wzbogaconych jonami żelaza bez użycia PEF zawierały około 266 mg Fe^{3+}/100 g suchej masy. Zastosowanie drożdży wzbogaconych w żelazo w warunkach PEF do przygotowania podpłomyków zwiększyło zawartość żelaza w tym produkcie do 386 mg Fe^{3+}/100 g suchej masy. Między próbkami wystąpiła znacząca różnica w potencjalnej biodostępności żelaza. Największą potencjalną biodostępnością żelaza odznaczały się podpłomyki z drożdżami P (10,8%), co skorelowane było z najwyższą zawartością tego pierwiastka w produkcie.

Inhibitorami wchłaniania żelaza są na przykład fityniany, czyli sole kwasu fitynowego występujące w roślinach; polifenole obecne w warzywach, owocach, niektórych zbożach i roślinach strączkowych, herbacie, kawie i winie. Wykazano również, że wapń

wpływa negatywnie na wchłanianie żelaza niehemowego i hemowego, odróżniając go od innych inhibitorów, które wpływają tylko na wchłanianie żelaza niehemowego (Hurrell i Egli, 2010). Jednym ze składników poprawiających wchłanianie żelaza jest kwas askorbinowy. Efekt ten wynika w dużej mierze z jego zdolności do redukcji żelaza (III) do żelaza (II) oraz jego zdolności do chelatowania żelaza (Pizarro i in., 2006).

W badaniach do produkcji podpłomyków użyto oczyszczonej mąki pszennej. W pracy nie analizowano zawartości fitynianu w mące i jego wpływu na biodostępność żelaza. Jednak dane literaturowe pokazują, że mąki podobne mąki użytej w badaniach charakteryzują się zawartością fitynianu ~100 mg/100g mąki (Hallberg, 1987).

W literaturze naukowej dostępnych jest wiele badań oceniających biodostępność żelaza z produktów zbożowych wzbogaconych w ten pierwiastek (Diego Quintaes i in., 2017). Jednak porównanie wyników jest utrudnione ze względu na różnice w metodologii badań. Na przykład Pizarro i in. (2006) przygotowali chleb wzbogacony siarczanem żelaza, który zawierał 47 mg tego pierwiastka w 1 kg. Autorzy podali, że średnia absorpcja żelaza z tego produktu oceniona metodą in vivo wyniosła 10,5%, co jest porównywalne z wynikami przedstawionymi w **PIII**.

Tabela 3. Zawartość żelaza i potencjalna biodostępność żelaza z podpłomyków

Podpłomyki	**Zawartość żelaza (mg) w 100 g suchej masy**	**Potencjalna biodostępność żelaza (%)**
C1	$2,96 \pm 0,54^{a}$	$5,86 \pm 0,12^{a}$
C2	$266,3 \pm 2,62^{b}$	$7,97 \pm 0,64^{b}$
P	$385,8 \pm 4,12^{c}$	$10,83 \pm 0,94^{c}$

C1 - drożdże bez dodatku jonów żelaza i bez PEF; C2 – drożdże z dodatkiem jonów żelaza i bez PEF; P - drożdże z dodatkiem jonów żelaza i PEF. Każda wartość jest średnią ± odchylenie standardowe (n=3). Wyniki z tą samą literą w kolumnie nie różnią się istotnie (p<0,05).

Akceptacja przez konsumentów produktów spożywczych zależy głównie od ich cech sensorycznych i właściwości prozdrowotnych. W związku z rosnącą świadomością zdrowego stylu życia rośnie również znaczenie produktów zbożowych zawierających pełne ziarno lub inne składniki funkcjonalne (Mir i in., 2014).

Wyniki oceny jakości podpłomyków za pomocą 5-punktowej skali ocen przedstawiono w tabeli 4. Jakość wszystkich podpłomyków oceniono jako dobrą, a podpłomyki wyprodukowane z drożdży P uzyskały najwyższą średnią ocenę.

Związki żelaza charakteryzujące się słabą rozpuszczalnością w prawidłowych stężeniach kwasu żołądkowego nie zaburzają właściwości sensorycznych pożywienia (Hurrell, 1997). Z drugiej strony, bardziej rozpuszczalne związki mogą powodować utlenianie tłuszczu (tj. zjełczenie) oraz zmianę koloru produktu w ciągu 6 miesięcy przechowywania. W przypadku oceny sensorycznej podpłomyków nie zanotowano metalicznego posmaku, który jest poważnym problemem dla produktów wzbogaconych solami żelaza.

Tabela 4. Wyniki oceny podpłomyków w 5-stopniowej skali ocen.

Cecha	Współczynnik ważkości	Podpłomyki		
		C1	C2	P
Kolor	0,3	$1,32 \pm 0,10^{b}$	$1,22 \pm 0,04^{a}$	$1,32 \pm 0,03^{b}$
Zapach	0,15	$0,39 \pm 0,01^{b}$	$0,37 \pm 0,02^{b}$	$0,33 \pm 0,01^{a}$
Struktura i konsystencja	0,15	$0,50 \pm 0,08^{a}$	$0,56 \pm 0,06^{a}$	$0,60 \pm 0,02^{a}$
Smak	0,4	$1,55 \pm 0,04^{a}$	$1,49 \pm 0,08^{a}$	$1,68 \pm 0,08^{b}$
Ocena ogólna		$3,76 \pm 0,12^{a}$	$3,64 \pm 0,11^{a}$	$3,93 \pm 0,03^{b}$

C1 - drożdże bez dodatku jonów żelaza i bez PEF; C2 – drożdże z dodatkiem jonów żelaza i bez PEF; P - drożdże z dodatkiem jonów żelaza i PEF. Każda wartość jest średnią ± odchylenie standardowe (n=3). Wyniki z tą samą literą w kolumnie nie różnią się istotnie (p<0,05).

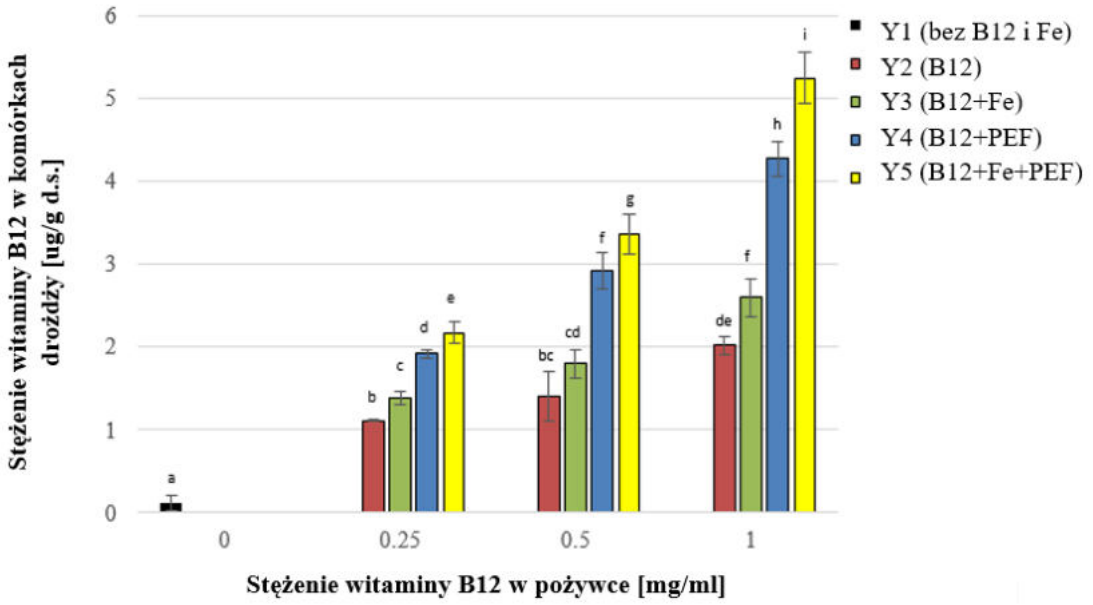
Zgodnie ze średnimi wynikami dla zapachu podpłomyków najwyższą notę 0,39 otrzymały podpłomyki z drożdżami C1 (niewzbogaconymi żelazem), ale różnice między punktacjami dla wszystkich podpłomyków były bardzo małe. Z tabeli 4 można wywnioskować, że barwa podpłomyków z dodatkiem drożdży C1 i P została oceniona najwyżej. Podpłomyki z dodatkiem drożdży P barwą przypominały produkty pełnoziarniste, które ze względu na rosnącą świadomość konsumentów na temat zdrowego trybu życia są bardziej akceptowalne i pożądane. Podpłomyki z dodatkiem drożdży P uzyskały najwyższe noty za strukturę i konsystencję.

5.7. Wybrane właściwości płatków drożdżowych z dodatkiem drożdży wzbogaconych jonami żelaza i witaminą B_{12} za pomocą PEF

Publikacja **PIV** dotyczy otrzymywania kolejnego produktu z drożdży wzbogaconych w jony żelaza z użyciem PEF. Dodatkowo do drożdży wprowadzono również witaminę B_{12}. Przeprowadzono również analizę wartości odżywczej oraz potencjalnej biodostępności

żelaza i witaminy B_{12} z płatków drożdżowych wykonanych z drożdży wzbogaconych jonami żelaza i witaminą B_{12} dwoma metodami: tylko poprzez dodanie soli żelaza i witaminy B_{12} do pożywki oraz poprzez dodatkowe zwiększenie akumulacji poprzez działanie PEF.

W pierwszym etapie badań PEF o parametrach optymalnych dla akumulacji jonów żelaza został wykorzystany do zwiększenia akumulacji witaminy B_{12} w komórkach drożdży. Wykazano, że przy niskim stężeniu witaminy B_{12} 0,25 mg/ml pożywki zastosowane parametry PEF nie miały wpływu na jej akumulację w komórkach *Saccharomyces cerevisiae* (rys. 17). Istotny wpływ PEF zaobserwowano dla stężenia witaminy B_{12} 1 mg/ml pożywki. Zawartość witaminy B_{12} w próbce Y5 (drożdże wzbogacone w 1 mg witaminy B_{12}/ml pożywki i 200 µg Fe^{3+}/ml pożywki z użyciem PEF) była o 50% wyższa niż w próbce Y3 (drożdże wzbogacone o to samo stężenie obu substancji, ale bez PEF). Z tego względu do kolejnych etapów eksperymentu postanowiono wykorzystać stężenie 1 mg/ml pożywki. Dodatkowo zaobserwowano, że drożdże Y3 i Y5 (odpowiednio 2,6 µg/g s.m. oraz 5,24 µg/g s.m.) akumulowały więcej witaminy B_{12} niż drożdże Y2 i Y4 (odpowiednio 1,8 µg/g s.m. oraz 4,34 µg/g s.m.). Możliwym wyjaśnieniem jest to, że obecność jonów żelaza ma stabilizujący wpływ na tę witaminę (Heep i Taterra, 2015). Dla porównania Jach i in. (2020) uzyskali biomasę drożdży *Yarrowia lipolytica* A-101 wzbogaconych witaminą B_{12} (bez użycia PEF) w ilości 9 µg witaminy B_{12} na 100 g suchej biomasy.



Rysunek 17. Wpływ stężenia witaminy B_{12} w pożywce na jej akumulację w komórkach drożdży. Czarne słupki (Y1) - hodowla kontrolna bez jonów żelaza, witaminy B_{12} i PEF; czerwone słupki (Y2) - hodowle z witaminą B_{12} i bez PEF, zielone słupki (Y3) - hodowle z dodatkiem witaminy B_{12} i jonów żelaza (200 µg Fe^{3+}/ml pożywki) i bez PEF, niebieskie słupki (Y4) - hodowle z B_{12} i z PEF (napięcie 1500 V, szerokość impulsu 10 µs, czas działania PEF 10 min, liczba impulsów 600, po 22 h hodowli), żółte słupki (Y5) – hodowle z witaminą B_{12} i jonami żelaza (200 µg Fe^{3+}/ml pożywki) i z PEF (napięcie 1500 V, szerokość impulsu 10 µs, czas działania PEF 10 min, liczba impulsów 600, po 22 h hodowli). Każda wartość jest średnią ± odchylenie standardowe (n=3). Słupki z tą samą literą (a - i) nie różnią się istotnie (p<0,05)

W literaturze do niedawna brakowało doniesień o zastosowaniu PEF do wzbogacania drożdży w witaminy. Ostatnio Nowosad i in. (2022) zastosowali tę metodę do zwiększenia akumulacji witaminy C w drożdżach *S. cerevisiae* za pomocą PEF. Najwyższą akumulację witaminy C w komórkach (ok. 1,3 mg/g suchej masy) uzyskano, gdy 20-godzinną hodowlę drożdży potraktowano PEF przy optymalnych parametrach: napięcie 1000 V, szerokość impulsu 10 µs, czas działania PEF 20 min oraz liczba impulsów 1200.

Drożdże można stosować w profilaktyce i leczeniu różnych dolegliwości i niedoborów, gdyż są bogatym źródłem aminokwasów, białek, minerałów takich jak chrom, selen, cynk, miedź, żelazo, mangan, magnez oraz witaminy z grupy B (Jach i in., 2018). W tabeli 5 przedstawiono wartość odżywczą i indeks glikemiczny płatków wytworzonych z drożdży z kultur niesuplementowanych oraz wzbogaconych w jony żelaza i witaminę B_{12} dwoma różnymi metodami. Zawartość węglowodanów w płatkach wahała się od 32-34%. Próbki C1 i C2 miały wyższą zawartość białka niż P. Najwyższą zawartość

tłuszczu stwierdzono w próbce C1. Próbki płatków drożdżowych różniły się istotnie zawartością popiołu, co było spowodowane wyższą zawartością żelaza w próbkach wzbogaconych o ten pierwiastek. Ilość węglowodanów, tłuszczów i białek wpływała na kaloryczność produktów. Stwierdzono statystycznie istotne różnice w wartości kalorycznej płatków drożdżowych z najwyższym wynikiem dla próbki C1. IG płatków drożdżowych wynosił około 42, dzięki czemu mogą stanowić uzupełnienie diety osób z insulinoopornością i cukrzycą.

Tabela 5. Skład odżywczy, kaloryczność i indeks glikemiczny (IG) płatków drożdżowych (zawartość podawana w przeliczeniu na suchą masę) wyprodukowanych z drożdży niewzbogaconych i wzbogaconych jonami żelaza i witaminą B_{12}

Płatki drożdżowe	Białko (%)	Węglowodany (%)	Tłuszcz (%)	Popiół (%)	Wartość kaloryczna (kcal/100 g)	Glycemic index (GI)
C1	53,02±0,17^{b}	33,79±0,28^{b}	2,70±0,14^{c}	6,38±0,04^{a}	387,97±0,89^{c}	42,12±0,11^{a}
C2	52,66±0,33^{b}	31,94±0,12^{a}	2,50±0,00^{b}	8,81±0,24^{b}	377,23±0,96^{b}	42,09±0,09^{a}
P	49,25±0,10^{a}	31,99±0,37^{a}	2,35±0,07^{a}	12,58±0,19^{c}	361,43±0,39^{a}	41,97±1,09^{a}

C1 – płatki drożdżowe bez dodatku jonów żelaza i witaminy B_{12} oraz bez PEF; C2 – płatki drożdżowe z dodatkiem jonów żelaza (200 µg Fe^{3+}/ml pożywki) i witaminy B_{12} (1 mg witaminy B_{12}/ml pożywki) i bez PEF; P – płatki drożdżowe z dodatkiem jonów żelaza (200 µg Fe^{3+}/ml pożywki) oraz witaminy B_{12} (1 mg witaminy B_{12}/ml pożywki) i PEF. Każda wartość jest średnią ± odchylenie standardowe (n=3). Wyniki z tą samą literą w kolumnie nie różnią się istotnie (p<0,05).

Produkty pochodzenia zwierzęcego są źródłem witaminy B_{12} w diecie. Średnia biodostępność tej witaminy u zdrowych ludzi z mięsa drobiowego waha się od 61 do 66% (Watanabe i in., 2007). Bioaktywne formy witaminy B_{12} nie występują w produktach roślinnych. Niektóre pokarmy roślinne, takie jak wodorosty i grzyby, zawierają analogi witaminy B_{12} (Watanabe i Bito, 2018).

Tabela 6 przedstawia zawartość żelaza i witaminy B_{12} oraz potencjalną biodostępność tych związków z płatków drożdżowych. Potencjalna biodostępność witaminy B_{12} z płatków drożdżowych wynosiła około 3,5% w próbce C2, natomiast w próbce P - około 4,3%. Wyższa biodostępność witaminy B_{12} w próbce P może być spowodowana wyższą zawartością witaminy B_{12} w tej próbce niż w C2. W naszym badaniu płatki drożdżowe zawierały o 82,5% mniej witaminy B_{12} niż drożdże świeże (5,25 µg/g suchej masy drożdży i 0,92 µg/g suchej masy płatków drożdżowych). Różnica w zawartości witaminy B_{12} między tymi produktami jest prawdopodobnie spowodowana wysoką temperaturą procesu

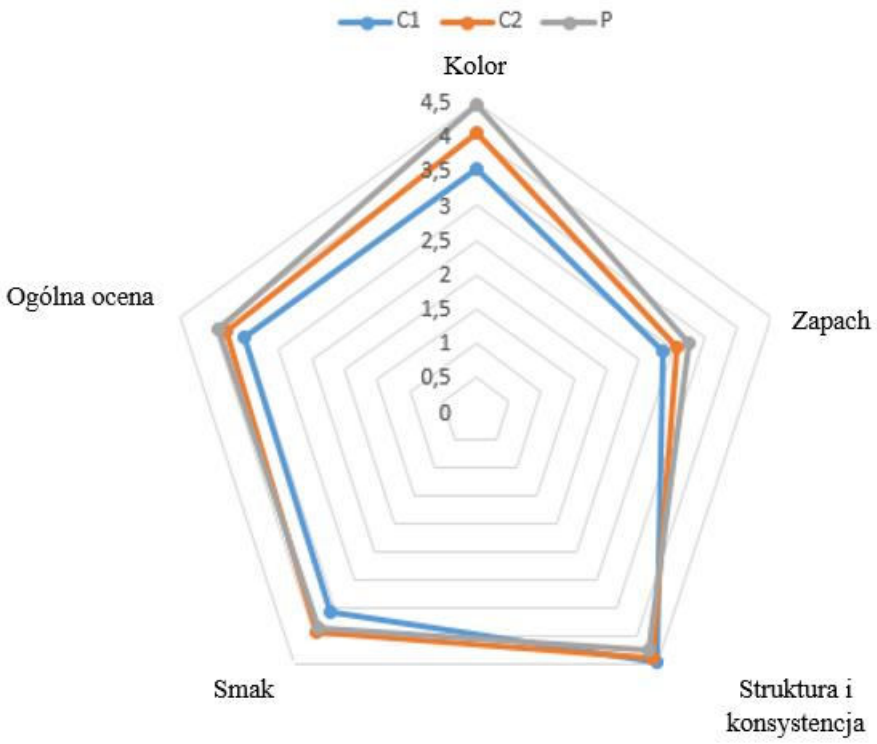
przygotowania płatków drożdżowych (Bajaj i Singhal, 2021). Płatki drożdżowe C1 i C2 zawierały odpowiednio około 12 mg i ok. 1420 mg żelaza w 100 g suchej masy. Zastosowanie drożdży wzbogaconych w żelazo w warunkach PEF do przygotowania płatków drożdżowych zwiększyło zawartość jonów żelaza w płatkach do prawie 3600 mg /100 g suchej masy. Potencjalna biodostępność żelaza z płatków drożdżowych wynosi około 6,8% dla próbki C2 i około 10% dla próbki P.

Tabela 6. Zawartość żelaza i witaminy B_{12} w 100 g suchej masy oraz potencjalna biodostępność żelaza z płatków drożdżowych

Płatki drożdżowe	Zawartość żelaza (mg/100 g)	Potencjalna biodostępność żelaza (%)	Zawartość witaminy B_{12} (µg/100 g)	Potencjalna biodostępność witaminy B_{12} (%)
C1	11,98±1,01[a]	5,16±0,60[a]	1,03±0,29[a]	0,00[a]
C2	1424,42±0,78[b]	6,77±0,36[b]	64,90±3,18[b]	3,53±0,76[b]
P	3593,10±4,33[c]	10,13±0,08[c]	92,42± 3,91[c]	4,31±0,44[c]

C1 – płatki drożdżowe bez dodatku jonów żelaza i witaminy B_{12} oraz bez PEF; C2 – płatki drożdżowe z dodatkiem jonów żelaza (200 µg Fe^{3+}/ml pożywki) i witaminy B_{12} (1 mg witaminy B_{12}/ml pożywki) i bez PEF; P – płatki drożdżowe z dodatkiem jonów żelaza (200 µg Fe^{3+}/ml pożywki) oraz witaminy B_{12} (1 mg witaminy B_{12}/ml pożywki) i PEF. Każda wartość jest średnią ± odchylenie standardowe (n=3). Wyniki z tą samą literą w kolumnie nie różnią się istotnie (p<0,05).

Płatki drożdżowe oceniono sensorycznie. Wyniki oceny ogólnej dla płatków drożdżowych P i C2 były najwyższe i nie różniły się istotnie (Rys. 16). Najniżej oceniono płatki drożdżowe C1. Barwa została oceniona wyżej dla produktu P niż C1 i C2 (nie stwierdzono statystycznie istotnych różnic między tymi dwiema próbkami, $p < 0,05$). Statystycznie istotne różnice stwierdzono między próbkami P, C2 i C1. Różnice te wynikały z ciemnoczerwonej barwy płatków C2 i P spowodowanej obecnością witaminy B_{12}, która ma postać czerwonych kryształków. Nie stwierdzono istotnych różnic pomiędzy płatkami drożdżowymi w takich cechach jak: struktura, konsystencja i smak. Jakość płatków drożdżowych C1 oceniono jako zadowalającą, natomiast płatków drożdżowych C2 i P jako dobrą. Obecność żelaza nie zmieniła właściwości sensorycznych produktu.



Rysunek 18. Wyniki oceny płatków drożdżowych metodą 5-punktową. C1 – płatki drożdżowe bez dodatku jonów żelaza i witaminy B_{12} bez PEF; C2 – płatki drożdżowe z dodatkiem jonów żelaza (200 µg Fe^{3+}/ml pożywki) i witaminy B_{12} (1 mg witaminy B_{12}/ml pożywki) bez PEF; P – płatki drożdżowe z dodatkiem jonów żelaza (200 µg Fe^{3+}/ml pożywki) i witaminy B_{12} (1 mg witaminy B_{12}/ml pożywki) oraz PEF. Każda wartość to średnia ± odchylenie standardowe (n=3)

6. Podsumowanie i wnioski

Postawione na wstępie hipotezy badawcze zweryfikowano przeprowadzając szereg eksperymentów na drożdżach wzbogaconych w jony żelaza za pomocą pulsacyjnego pola elektrycznego. Znaczące różnice w akumulacji jonów żelaza między próbkami kontrolnymi (C1, C2) a próbkami poddanymi działaniu PEF (P) wskazują na możliwość wykorzystania pulsacyjnego pola elektrycznego jako sposobu na suplementację drożdży w wybrane składniki mineralne. W rozprawie doktorskiej wykazano, że drożdże *Saccharomyces cerevisiae* mogą stanowić surowiec do produkcji żywności potencjalnie funkcjonalnej o pożądanych cechach fizykochemicznych. Przeprowadzone badania rozszerzyły wiedzę z zakresu wykorzystania drożdży jako nośników składników mineralnych i witamin.

Przeprowadzone badania pozwoliły na sformułowanie następujących wniosków:

1. Zastosowanie pulsacyjnego pola elektrycznego prowadzi do zwiększenia akumulacji jonów żelaza komórkach drożdży.
2. Zastosowanie optymalnych warunków pulsacyjnego pola elektrycznego powoduje wzrost akumulacji jonów żelaza w komórkach *Saccharomyces cerevisiae* o 157% w porównaniu z próbką wzbogaconą żelazem bez PEF. Po zastosowaniu następujących warunków obróbki PEF: napięcie 1500 V, szerokość impulsu 10 μs, czas działania PEF 20 min i liczba impulsów 1200, akumulacja jonów żelaza w komórkach drożdży z 20 h hodowli osiąga wartość maksymalną 48,01 mg Fe^{3+}/g suchej masy.
3. Zastosowanie pulsacyjnego pola elektrycznego do zwiększenia akumulacji jonów żelaza nie powoduje istotnego zmniejszenia produkcji biomasy oraz żywotności komórek drożdży *Saccharomyces cerevisiae*.
4. Aktywność fermentacyjna drożdży wzbogaconych w jony żelaza bez udziału PEF, jak i z zastosowaniem PEF jest niższa niż drożdży niewzbogaconych.
5. Drożdże wzbogacone w jony żelaza (oraz dodatkowo w witaminę $B_{12)}$ mogą zostać wykorzystane do produkcji podpłomyków i płatków drożdżowych charakteryzujących się ok. 10% potencjalną biodostępnością tego pierwiastka.

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8. Oświadczenia o procentowym udziale autorów w opracowaniu publikacji

Karolina Nowosad
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Lublin, 15.01.2023 r.

OŚWIADCZENIE O UDZIALE AUTORÓW W OPRACOWANIU PUBLIKACJI

Oświadczenie dotyczy publikacji pt.:

The application of PEF technology in food processing and human nutrition
Tytuł artykułu

Journal of Food Science and Technology, 2021, 58(2), 397-411. https://doi.org/10.1007/s13197-020-04512-4
Nazwa czasopisma, rok, numer, strony, DOI

Karolina Nowosad, Monika Sujka, Urszula Pankiewicz, Radosław Kowalski
Autorzy

Lp.	Nazwisko i imię autora	Afiliacja	Indywidualny wkład w powstanie publikacji	Podpis autora
1.	Karolina Nowosad**	Katedra Analizy i Oceny Jakości Żywności, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Udział w opracowaniu koncepcji publikacji, gromadzenie literatury, przygotowanie i redagowanie manuskryptu	
2.	Monika Sujka*	Katedra Analizy i Oceny Jakości Żywności, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Udział w opracowaniu koncepcji publikacji, odpowiedzi na recenzje. redagowanie publikacji	MSujka
3.	Urszula Pankiewicz	Katedra Analizy i Oceny Jakości Żywności, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Udział w opracowaniu koncepcji publikacji, redagowanie publikacji	
4.	Radosław Kowalski	Katedra Analizy i Oceny Jakości Żywności, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Udział w opracowaniu koncepcji publikacji.	

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OŚWIADCZENIE O UDZIALE AUTORÓW W OPRACOWANIU PUBLIKACJI

Oświadczenie dotyczy publikacji pt.:

Pulsed Electric Field (PEF) Enhances Iron Uptake by the Yeast *Saccharomyces cerevisiae*
Tytuł artykułu

Biomolecules, 2021, 11(6), 850; https://doi.org/10.3390/biom11060850
Nazwa czasopisma, rok, numer, strony, DOI

Karolina Nowosad, Monika Sujka, Urszula Pankiewicz, Damijan Miklavčič, Marta Arczewska
Autorzy

Lp.	Nazwisko i imię autora	Afiliacja	Indywidualny wkład w powstanie publikacji	Podpis autora
1.	Karolina Nowosad**	Katedra Analizy i Oceny Jakości Żywności, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Udział w opracowaniu koncepcji badań, wykonanie analiz laboratoryjnych (optymalizacja parametrów PEF, oznaczenie biomasy i żywotności komórek drożdży oraz właściwości fermentacyjnych), opracowanie wyników i ich interpretacja, przygotowanie manuskryptu.	
2.	Monika Sujka*	Katedra Analizy i Oceny Jakości Żywności, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Udział w opracowaniu koncepcji badań oraz metodyki badawczej, udział w przygotowaniu próbek do analizy mikroskopowej, interpretacja wyników, odpowiedzi na recenzje, redagowanie manuskryptu.	
3.	Urszula Pankiewicz	Katedra Analizy i Oceny Jakości Żywności, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Udział w opracowaniu metodyki badań (optymalizacja parametrów PEF).	
4.	Damijan Miklavčič	Laboratorium Biocybernetyki, Wydział Elektryczny, Uniwersytet w Lublanie	Udział w opracowaniu metodyki badań (dobór warunków działania PEF), redagowanie manuskryptu.	Damijan Miklavči č Digitally signed by Damijan Miklavčič Date: 2023.01.15 16:18:02 +01'00'
5.	Marta Arczewska	Katedra Biofizyki, Wydział Biologii Środowiskowej, UP w Lublinie	Wykonanie analizy FTIR oraz opracowanie wyników i ich interpretacja, redagowanie manuskryptu.	

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OŚWIADCZENIE O UDZIALE AUTORÓW W OPRACOWANIU PUBLIKACJI

Oświadczenie dotyczy publikacji pt.:

The Use of Iron-Enriched Yeast for the Production of Flatbread
Tytuł artykułu

Molecules, 2021, 26(17), 5204; https://doi.org/10.3390/molecules26175204
Nazwa czasopisma, rok, numer, strony, DOI

Karolina Nowosad, Monika Sujka
Autorzy

Lp.	Nazwisko i imię autora	Afiliacja	Indywidualny wkład w powstanie publikacji	Podpis autora
1.	Karolina Nowosad**	Katedra Analizy i Oceny Jakości Żywności, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Udział w opracowaniu koncepcji badań i metodyki, wykonanie badań laboratoryjnych, opracowanie wyników i ich interpretacja, przygotowanie i redagowanie manuskryptu, odpowiedzi na recenzje	
2.	Monika Sujka*	Katedra Analizy i Oceny Jakości Żywności, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Udział w opracowaniu koncepcji badań i metodyki oraz interpretacji wyników, redagowanie manuskryptu, odpowiedzi na recenzje	

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Lublin, 15.01.2023 r.

OŚWIADCZENIE O UDZIALE AUTORÓW W OPRACOWANIU PUBLIKACJI

Oświadczenie dotyczy publikacji pt.:

Preparation of yeast flakes enriched with iron and vitamin B12 using a pulsed electric field technology

Tytuł artykułu

Journal of Food Process Engineering, 2022, https://doi.org/10.1111/jfpe.14245

Nazwa czasopisma, rok, numer, strony, DOI

Karolina Nowosad, Monika Sujka, Jakub Wyrostek
Autorzy

Lp.	Nazwisko i imię autora	Afiliacja	Indywidualny wkład w powstanie publikacji	Podpis autora
1.	Karolina Nowosad**	Katedra Biotechnologii, Mikrobiologii i Żywienia Człowieka, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Udział w opracowaniu koncepcji badań i metodyki, wykonanie badań laboratoryjnych, opracowanie wyników i ich interpretacja, przygotowanie i redagowanie manuskryptu, odpowiedzi na recenzje.	
2.	Monika Sujka*	Katedra Analizy i Oceny Jakości Żywności, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Udział w opracowaniu koncepcji badań i metodyki oraz interpretacji wyników, odpowiedzi na recenzje, redagowanie manuskryptu.	
3.	Jakub Wyrostek	Katedra Analizy i Oceny Jakości Żywności, Wydział Nauk o Żywności i Biotechnologii, UP w Lublinie	Analiza chromatograficzna	

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9. Zestawienie dorobku naukowego

UNIWERSYTET PRZYRODNICZY w Lublinie

BIBLIOTEKA GŁÓWNA UNIWERSYTETU PRZYRODNICZEGO W LUBLINIE

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Biblioteka Główna UP w Lublinie
Baza publikacji Pracowników Uniwersytetu Przyrodniczego
Raport autora – mgr inż. Karolina Nowosad

1. Publikacje w czasopismach naukowych

1.1. Publikacje w czasopiśmie naukowym posiadającym Impact Factor IF

Lp	Opis bibliograficzny	IF	Pkt. MNiSW
1.	**Preparation of yeast flakes enriched with iron and vitamin B12 using a pulsed electric field technology.** [AUT.] KAROLINA NOWOSAD, [AUT. KORESP.] MONIKA SUJKA, [AUT.] JAKUB WYROSTEK. *J. Food Process Eng.* 2023 Volume 46 Issue 2 Article number e14245, il., bibliogr., sum. DOI: 10.1111/jfpe.14245	2,889	100,00
2.	**Accumulation of vitamin C in yeast under pulsed electric field (PEF) conditions.** [AUT.] KAROLINA NOWOSAD, [AUT. KORESP.] MONIKA SUJKA, [AUT.] EWELINA ZIELIŃSKA, URSZULA PANKIEWICZ. *Appl. Sci.-Basel* 2022 Vol. 12 Iss. 20 Article number 10206, il., bibliogr., sum. DOI: 10.3390/app122010206	2,838	100,00
3.	**Pulsed electric field (PEF) enhances iron uptake by the yeast *Saccharomyces cerevisiae*.** [AUT.] KAROLINA NOWOSAD, [AUT. KORESP.] MONIKA SUJKA, [AUT.] U[RSZULA] PANKIEWICZ, DAMIJAN MIKLAVČIČ, MARTA ARCZEWSKA. *Biomolecules* 2021 Vol. 11 Iss. 6 Article number 850, il., bibliogr.,sum. DOI: 10.3390/biom11060850	6,064	100,00
4.	**The application of PEF technology in food processing and human nutrition.** [AUT.] KAROLINA NOWOSAD, [AUT. KORESP.] MONIKA SUJKA, [AUT.] URSZULA PANKIEWICZ, RADOSŁAW KOWALSKI. *J. Food Sci. Technol.* 2021 Vol. 58 Issue 3 s. 397 - 411, il., bibliogr., sum. DOI: 10.1007/s13197-020-04512-4	3,117	70,00
5.	**The use of iron-enriched yeast for the production of flatbread.** [AUT.] KAROLINA NOWOSAD, [AUT. KORESP.] MONIKA SUJKA. *Molecules (Basel, Online)* 2021 Vol. 26 Issue 17 Article number 5204, il., bibliogr., sum. DOI: 10.3390/molecules26175204	4,927	140,00
	Suma:	19,835	510,00

1.2 Publikacja w czasopiśmie naukowym nieposiadającym IF

Lp	Opis bibliograficzny	Punkty ministerialne
1.	**Zastosowanie pulsacyjnego pola elektrycznego (PEF) jako zabiegu wspomagającego ekstrakcję (Application of the pulsed electric field (PEF) as the extraction assistant treatment).** [AUT.] KAROLINA NOWOSAD. *Wiad. Chem.* 2022 Vol. 76 Nr 3/4 s. 207-220, bibliogr., sum. DOI: 10.53584/wiadchem.2022.3.6	20,00
2.	**Effect of various types of intermittent fasting (IF) on weight loss and improvement of diabetic parameters in human.** [AUT.] KAROLINA NOWOSAD, [AUT. KORESP.] MONIKA SUJKA. *Curr. Nutr. Rep.* 2021 Vol. 10 s. 146-154, il., bibligr. sum. DOI: 10.1007/s13668-021-00353-5	70,00
3.	**Niedobór witaminy D w wybranych jednostkach chorobowych (Vitamin D deficiency in selected diseases).** [AUT.] KAROLINA NOWOSAD. *Nauki Przyr. i Med.(Lub.)* 2021 nr 2 (32) s. 3-12, il., bibliogr., streszcz., sum.	5,00
4.	**Rola diety i stylu życia w leczeniu insulinooporności.** [AUT.] KAROLINA NOWOSAD. *Kosmos (Warsz.)* 2021 T. 70 Nr 4 s. 731-739, bibliogr., sum. DOI: 10.36921/kos.2021_2801	20,00
5.	**Wpływ metody suszenia na właściwości przeciwutleniające ziela bazylii, mięty oraz pietruszki.** [AUT.] KAROLINA NOWOSAD, MONIKA SUJKA. *Żywn. Nauka Technol. Jakość (2016-)* 2021 Vol. 28 nr 4 (129) s. 57-68, il. bibliogr., streszcz., sum. DOI: 10.15193/zntj/2021/129/400	20,00
6.	**Witamina D: metabolizm, funkcje oraz toksyczność (Vitamin D: metabolism, function and toxicity).** [AUT.] KAROLINA NOWOSAD. *Nauki Przyr. i Med.(Lub.)* 2020 nr 4 (30) s. 46-53, il., bibliogr., streszcz., sum.	5,00
7.	**Wpływ bisfenolu A na zdrowie człowieka (Effect of bisphenol A on human health).** [AUT.] KAROLINA NOWOSAD. *Wiad. Chem.* 2020 Vol. 74 Nr 5/6 s. 411-422, bibliogr., sum.	20,00
8.	**Charakterystyka najzdrowszych diet na świecie (Characteristics of the healthiest diets in the world).** [AUT.] KAROLINA NOWOSAD. *Nauki Przyr. i Med.(Lub.)* 2019 nr 4 (26) s. 22-32, il., bibliogr., streszcz., sum.	5,00
9.	**Niekonwencjonalne metody ekstrakcji - ekstrakcja ekologiczna (Unconventional methods of extractions - green extraction).** [AUT. KORESP.] KAROLINA NOWOSAD, [AUT.] MONIKA SUJKA. *Wiad. Chem.* 2019 Vol. 73 Nr 9/10 s. 465-479, bibliogr., sum.	20,00
10.	**Otyłość a niedobór witaminy D (Obesity and vitamin D deficiency).** [AUT.] KAROLINA NOWOSAD. *Nauki Przyr. i Med.(Lub.)* 2019 nr 4 (26) s. 12-20, il., bibliogr., streszcz., sum.	5,00
11.	**Wpływ wielonienasyconych kwasów tłuszczowych na zdrowie człowieka (The effect of polyunsaturated fatty acids on human health).** [AUT.] KAROLINA NOWOSAD. *Nauki Przyr. i Med.(Lub.)* 2019 nr 2 (24) s. 20-28, il., bibliogr., streszcz., sum.	5,00
12.	**Porous starch and its application in drug delivery systems.** [AUT. KORESP.] MONIKA SUJKA, [AUT.] URSZULA PANKIEWICZ, RADOSŁAW KOWALSKI, KAROLINA NOWOSAD, AGNIESZKA NOSZCZYK-NOWAK. *Polim. Med.* 2018 Vol. 48 T. 48 Nr 1 s. 25-29, il., bibliogr. DOI: 10.17219/pim/99799	9,00
	Suma:	204,00

2. Monografie naukowe

2.1 Autorstwo rozdziału w monografii naukowej

Lp	Opis bibliograficzny	Punkty ministerialne
1.	**Ryzyko niedoborów witaminy B12 i żelaza w diecie wegetariańskiej i wegańskiej (Risk of vitamin B12 and iron deficiency in vegetarian and vegan diets).** [AUT.] KAROLINA NOWOSAD. W: Badania i Rozwój Młodych Naukowców w Polsce. Żywność i żywienie (sierpień 2021) Poznań 2021, Młodzi Naukowcy, s. 54-58, 978-83-66743-20-5.	5,00
2.	**Zastosowanie pulsacyjnego pola elektrycznego do bioakumulacji jonów metali w biomasie komórkowej drożdży i bakterii (Application of a pulsed electric field for the bioaccumulation of metal ions in the cell biomass of yeast and bacteria).** [AUT.] KAROLINA NOWOSAD. W: Badania i Rozwój Młodych Naukowców w Polsce. Żywienie i żywność Poznań 2021, Młodzi Naukowcy, s. 75-80, 978-83-66392-91-5.	5,00
3.	**Zastosowanie pulsacyjnego pola elektrycznego w przemyśle spożywczym (Application of the pulsed electric field in the food industry).** [AUT.] KAROLINA NOWOSAD. W: Badania i Rozwój Młodych Naukowców w Polsce. Żywienie i żywność Poznań 2021, Młodzi Naukowcy, s. 69-74, 978-83-66392-91-5.	5,00
4.	**Analiza fizykochemiczna i organoleptyczna miodów pitnych dostępnych na rynku (Physicochemical and organoleptic analysis of meads available on the market).** [AUT.] KAROLINA NOWOSAD, MONIKA SUJKA. W: Żywność i żywienie / Redakcja naukowa Marcin Baran, Jędrzej Nyćkowiak Poznań 2019, Młodzi Naukowcy, s. 54-61, il., bibliogr., streszcz, 978-83-66139-99-2.	5,00
5.	**Yerba mate –charakterystyka, właściwości prozdrowotne i antyzdrowotne (Yerba mate –characteristics, pro-health and anti-health properties).** [AUT.] KAROLINA NOWOSAD, MONIKA SUJKA. W: Wybrane właściwości roślin- najnowsze doniesienia / Redakcja: Alicja Danielewska, Kamil Maciąg Lublin 2019, Wydawnictwo Naukowe TYGIEL Sp. z o. o, s. 126-134, bibliogr.,sum, 978-83-65932-94-5.	20,00
6.	**Zwyczaje żywieniowe dzieci w Polsce w różnych środowiskach.** [AUT.] KAROLINA NOWOSAD, MONIKA SUJKA. W: Biogospodarka i zagrożenia środowiska. T.1 / Monografia pod redakcją Bożeny Nowakowicz-Dębek i Witolda Chabuza [Radom 2019], Instytut Naukowo-Wydawniczy Spatium, s. 137-144, il., bibliogr, 978-83-66017-72-6.	20,00
	Suma:	60,00

3. Inne

3.1 Materiały konferencyjne

Lp	Opis bibliograficzny
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REVIEW ARTICLE

The application of PEF technology in food processing and human nutrition

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Abstract During the last decades, many novel techniques of food processing have been developed in response to growing demand for safe and high quality food products. Nowadays, consumers have high expectations regarding the sensory quality, functionality and nutritional value of products. They also attach great importance to the use of environmentally-friendly technologies of food production. The aim of this review is to summarize the applications of PEF in food technology and, potentially, in production of functional food. The examples of process parameters and obtained effects for each application have been presented.

Keywords Pulsed electric field · Food processing · Functional food

Introduction

Development of innovative food processing methods can increase the competitiveness of the food industry by improving product quality, introducing new products to the market and reducing production costs (Tiwari et al. 2009). Incorporation of pulsed electric field (PEF) technology into food production was supported by the growing consumer interest in food of high nutritional value, the demand for fresh-like products as well as food produced with the use of environmentally friendly methods (Evans and Cox 2006; Soliva-Fortuny et al. 2009). The studies showed that despite the fact that consumers have rather conservative approach and it is not always easy for them to see the benefits of novel processing technologies, they appreciate the naturalness, improved taste and high nutritional value of the products subjected to PEF (Nielsen et al. 2009; Sonne et al. 2012). As suggested by the results of studies conducted in different countries, providing detailed and reliable information about new technologies may be of key importance for increasing consumer acceptance of products obtained using novel food processing technologies (Lee et al. 2015; Galati et al. 2019; Maherani et al. 2016).

Despite many scientific studies on the principles and applications of PEF technology published so far and the fact that PEF was introduced into the food industry many years ago, this technology is still considered emerging. In the European Union there is no special legislation on food processed with PEF. In general the use of this technique is regulated by the Novel Food Regulation (EU) 2015/2283, but implementation of PEF into production does not automatically mean the food becomes "novel". According to Article 4 of Regulation (EC) No. 258/97, a food product can be considered as novel if the production process applied causes significant changes in its composition or structure influencing nutritional value, metabolism or level of undesirable substances. The studies showed that, for instance, in case of liquid products such as oils, juices and beverages containing juices no significant decreases in content of health-beneficial compounds have been observed as a result of PEF treatment (Guderjan et al. 2005; Zulueta et al. 2007; Salvia-Trujillo et al. 2011; Morales-De La Peña et al. 2012; Vallverdú-Queralt et al. 2012). The party who wants to market the food is responsible for clarifying its regulatory status with the national food authority body. Decision on food novelty is based on the procedures described in the Commission

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Implementing Regulation (EU) 2018/456 and safety assessment must be carried out as a part of the authorisation process. The use of novel processing technologies has the potential to reduce the environmental impact of food production and increase food safety, so their use is regulatory encouraged in the EU (Regulation (EU) 2015/2283). Regulations concerning novel foods exist also in Canada, New Zealand/Australia, China, and Brazil but the definition of "a novel food" may differ (Magnuson et al. 2013). In the United States, prior to 2002, the Food and Drug Administration considered pasteurization as a thermal treatment but in September 2004, the USDA National Advisory Committee on Microbiological Criteria for Foods (NACMCF) redefined the term "pasteurization" as "any process, treatment, or combination thereof, that is applied to food to reduce the most microorganism(s) of public health significance to a level that is not likely to present a public health risk under normal conditions of distribution and storage" allowing methods such as PEF to be used (NACMCF 2004). PEF has been used for the commercial pasteurization of juices in compliance with the mandates of FDA's juice HACCP regulations (21 C.F.R. 120). The juice processors also have to implement sanitation and Good Manufacturing Practices during production of juice and juice products. According to FDA the processes of production should meet a performance standard of 5 log reduction of the most resistant pathogen (FDA 2000).

Pasteurization of liquid foods still remains the main purpose of using PEF technology. The lethal effect of PEF on various vegetative bacteria, mold, and yeast can be strengthen by combining with other physical methods, such as UV radiation (Gachovska et al. 2008), high intensity light pulses (HILP) (Caminiti et al. 2011), ultrasound (Aadil et al. 2018), high pressure carbon dioxide (Pataro et al. 2010) and manothermosonication (Palgan et al. 2012).

General overview: advantages and disadvantages of PEF

PEF is a method that uses electric waves with high voltage amplitude. Short electrical impulses (from microseconds to milliseconds each) of high voltage (typically 10–80 kV/cm) are supplied to the product placed between the electrodes in the chamber (Deeth et al. 2008). Depending on the properties of the processed food product and the effects to be obtained, the process conditions such as electric field strength (kV/cm), pulse frequency, pulse width, shape of the pulse wave and exposure time (related to the flow rate and volume of fluid in the electrode chamber) can be modified suitably. For instance, the range of electric field strength 0.1–1 kV/cm causes reversible permeabilization of plant cells, 0.5–3 kV/cm—irreversible permeabilization of plant and animal tissue, 15–40 kV/cm—irreversible permeabilization of microbial cells (Tsong 1996).

In the last decade, one of the main fields of research in the scope of alternative energy-saving processes has been the application of PEF as a non-thermal method in food processing (Soliva-Fortuny et al. 2009). Nevertheless, it should be mentioned that the energy of the electric pulses generates heat due to Joule heating so cooling is necessary to maintain a low temperature of the processed product during PEF treatment. On the other hand, this phenomenon can be applied for a gentle preservation process. The combination of high temperature and PEF membrane electroporation improves also the inactivation efficiency (Jaeger et al. 2010b).

Most of the research on the use of PEF relates to inactivation of enzymes and microorganisms. High voltage impulses break the cell membrane making it permeable to small molecules, which causes the cells to begin to swell and break (Zimmerman 1986). PEF can be used for liquid and semi-solid products e.g. soups, liquid eggs or fruit juices (Qin et al. 1995). Fruit juices processed with this technology were introduced to the US market in 2005 (Ravishankar et al. 2008). In the case of solid products, PEF technology has found application mainly in potato processing industry. Potatoes can be subjected to PEF immediately after peeling and before the cutting step (Faridnia et al. 2015a) or in a form of slices. The effect of PEF is a change in the structural integrity of tissues, which results in more controlled release of intracellular compounds such as reducing sugars or amino acids involved in Maillard reactions, and therefore reduces acrylamide content in cooked or fried potato products (Jaeger et al. 2010a, b; Janositz et al. 2011; Genovese et al. 2019). Potatoes treated with PEF also have a more uniform color and absorb less oil during frying (Ignat et al. 2015; Liu et al. 2018a, b). Another effect of PEF is a softer texture that facilitates potato processing, e.g. cutting (Lebovka et al. 2004) and a significant decrease in drying time of potato discs (Fauster et al. 2018).

Although this technology has been investigated extensively and there are several dozen commercial PEF systems working around the world, the majority of the obtained results refer to the experiments carried out at laboratory scale. The pulsed electric field technology itself is generally considered to be safe for humans, because no dangerous chemical reactions have been detected (Frewer et al. 2011). However, the results of some studies indicate that electrode material constituents (e.g. Fe, Cr, Ni, Mn) are released to the liquid food samples due to corrosion (Roodenburg et al. 2005; Pataro et al. 2014). This problem may be overcome by application of carbon electrodes (Toepfl et al. 2004). According to Pataro et al. (2014),

some electrical parameters such as pulse frequency and the composition of the processed product (e.g. presence of halides) affect the amount of metal released from the electrodes. Undoubtedly, further research is needed to determine the optimal conditions for PEF treatment on an industrial scale, as well as electrode material and geometry, so that undesirable electrode reactions will be eliminated or at least minimized.

A typical PEF unit is composed of a few basic components: high-voltage pulse generator, treatment chamber, fluid-handling system, control and monitoring devices (Fig. 1). The first component supplies the high voltage pulses with required shape, duration and intensity. The generated pulses are applied to a pair of electrodes present in the treatment chamber and the treated product is placed between them. Depending on the type of the treated product (solid, semisolid, liquid, semiliquid), the treatment chambers can be divided into batch treatment chambers and continuous treatment chambers. The latter type is very convenient for industrial processes because allows liquid and semi-liquid products to be pumped through the chamber. The process is controlled by a central computer which is used for setting the parameters, controlling the operation of pump and gathering data from the probes placed inside the chamber (Barbosa-Canovas et al. 2004). In liquid products processed with PEF the serious problem is non-uniformity of electric field distribution inside the treatment chamber caused by its configuration, presence of bubbles/impurities and thermophysical properties of the product itself (Zhang et al. 1995). As a result some parts of the liquid volume can be undertreated (often in central or dead spaces) or overtreated (often in boundary regions). Achieving of electric field uniformity is particularly important in the case of cold pasteurization, because during this process all microorganisms present in the liquid should be exposed to the same electric field strength and the same number of pulses. To overcome this problem, treatment chambers with parallel plate electrode configurations or multiple PEF treatment chambers placed in series can be used (Buckow et al. 2013).

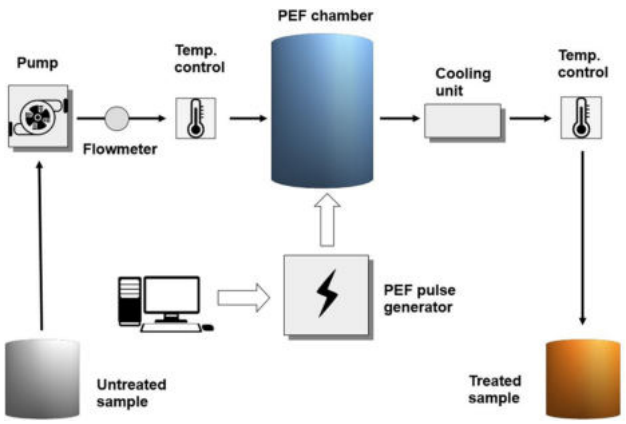

Fig. 1 A typical PEF unit used in food processing

Mechanism of pulsed electric field

The application of PEF on plant, animal or microbial cells disturbs transiently or permanently the integrity of cell membrane increasing its permeability, however the mechanism of PEF activity has not been fully understood. Until now, several theoretical models have been suggested, but there is still no evidence of PEF's action regimen at the cellular level (Soliva-Fortuny et al. 2009). Experimental evidence suggests that aqueous hydrophilic pores are formed as a consequence of rearrangements of the membrane components such as water and lipids, induced by long and intense electrical pulses (Weaver 2003). It is not possible to observe directly pores of sizes in nanometers with conventional methods e.g. electron microscopy. However, nowadays computational methods (molecular dynamics simulations) can be used to model the effect of electric field in cell membrane (Leach 2001) The simulations conducted by Tieleman (2004) have evidenced that the electroporation process takes place in two stages: (1) water molecules organized in single wire penetrate the hydrophobic core of the bilayer; (2) the water wires grow in length and expand into water-filled pores, which are later stabilized by reorganization of lipid molecules.

When a biological cell is considered as an electrolyte surrounded by an electrically insulted shell (the cytoplasm surrounded by the plasma membrane) and it is exposed to an external electric field, this results in occurrence of induced transmembrane voltage (Kotnik et al. 2010). Under physiological conditions there is a ionic gradient across the membrane resulting from the work of sodium–potassium pumps and potassium leak channels. Its value depends on cell type and typically ranges from − 80 to − 40 mV (Kotnik and Miklavčič 2006). Permeabilization of cell membrane is a local process and takes place when the transmembrane potential difference induced by electric field reaches 250 mV. This part of cell surface becomes highly permeable for small charged molecules such as DNA or metal ions (Teissié and Tsong 1981). Diffusion is observed mainly after the pulse and lasts for seconds and minutes (Gabriel and Teissie 1997). Permeabilization is dependent on field strength, pulse parameters (amplitude, duration, pulse number and repetition rate), membrane composition, surrounding media, temperature, cell size and shape and its orientation to the electric field lines (Valič et al. 2003). This phenomenon can be detected e.g. with the use of fluorescent indicators such as propidium iodide (PI)

(Sadik et al. 2013) or by means of electrical measurements (bio-impedance or micro-electrodes techniques) (Silve et al. 2011, 2017). The uptake of the indicator by the cells is the evidence of membrane permeabilization. When the operation of the electric field stops, the membrane defects become sealed, and the cells retain the introduced molecules or ions. It depends on the time of exposure and the intensity of the electric field. Resealing can last from a few second to several hours, depending on temperature. For example, at 37 °C the membrane defects close in a few seconds, at 4 °C in a few minutes and when cells are maintained on ice—several hours are needed. When the field strength exceeds the critical value significantly, the process is irreversible and can lead to cell destruction (Golzio et al. 2002).

Applications of PEF in food processing

The PEF technique was of interest already in the twentieth century. At the beginning of the 1990s, a milk pasteurization method was developed in which a low-frequency alternating electric field was used. In 1960 a German engineer Doevenspeck patented a method that used high-voltage electric waves to break down the structure of the cells of food materials (Toepfl et al. 2006). Processing factors such as electric field strength, pulse shape, pulse width, treatment time, pulse frequency and polarity, temperature, treatment in batch or continuous flow system are critical factors determining the efficiency of PEF technology in food processing. Optimization of PEF parameters is required for each specific application of pulsed electric field. Some examples are presented in Table 1.

Drying

Pre-treatment of the sample with PEF in order to destroy the cell structure reduces its resistance to diffusion and the mass and heat transfer rates between the cells and their surroundings increases (Barba et al. 2015). Research on the impact of initial PEF treatment on drying kinetics as well as changes in color and texture in sliced parsnip and carrot was carried out by Alam et al. (2018). The drying time was reduced to 28% at 70 °C and to 21% at 60 °C compared to the untreated samples. Wiktor et al. (2016) observed that drying time of the PEF-treated carrot samples was reduced up to 8.2%, the effective water diffusion coefficient increased up to 16.7%, and samples after drying exhibited higher lightness and redness in comparison to the intact tissue. Effect of a PEF pre-treatment on drying of onions was investigated by Ostermeier et al. (2018). The study revealed that a rising electric field strength up to 1.07 kV/cm caused an increase of the cell disintegration which facilitated the moisture release to the surface of the product. The higher diffusion led to a 30% reduction in drying time for PEF pre-treated onion samples dried at 45 °C. Telfser and Gómez Galindo (2019) studied the effect of reversible permeabilization as pre-treatment before air drying at 40 °C, vacuum drying and freeze drying of basil (*Ocimum basilicum* L.) leaves. The application of PEF shortened the drying time by 57% for air drying, 33% for vacuum drying and 25% for freeze drying. Samples which were PEF-treated and vacuum dried were found to be the closest to fresh leaves regarding colour and smell determined by sensory panel. Application of PEF accelerates also the drying of carrots, potatoes, apples, coconuts or paprika (Ade-Omowaye et al. 2001).

Freezing

Freezing food has one major disadvantage—the formation of ice crystals can destroy the tissue so that after thawing the products (for example soft fruits, leafy vegetables) lose their shape and become sodden. In this form they are not accepted by the consumers. It has been demonstrated that pulsed electric fields can be used to improve freezing tolerance of baby spinach leaves. PEF was applied with vacuum impregnation in the presence of cryoprotectants such as trehalose, sucrose, glucose, and fructose. The combination of these methods caused that leaf cells remained viable and the leaves retained turgor after the freezing and thawing cycle (Demir et al. 2018). Carrot discs treated with PEF after soaking in different cryoprotectant and texturizing agents had higher firmness after thawing than control sample (Shayanfar et al. 2014). Similar studies were carried out for potato strips. The results showed that PEF treatment by itself (without texturizing and antifreeze agents) was not a suitable pre-treatment method but when it was applied with $CaCl_2$ and trehalose potato strips maintained structural integrity, firmness and colour after thawing (Shayanfar et al. 2013). Interestingly, there was no improvement in texture of strawberries frozen and thawed after the application of PEF coupled with vacuum infusion and cryoprotectants. However, such treatment enhanced the color retention of thawed fruits (Velickova et al. 2018).

Application of PEF technology together with freezing or freeze-drying affects freezing time and rate. For example, the study of Jalté et al. (2009) showed that PEF pre-treatment can reduce the freezing time, increase the rate of freeze-drying and improve quality of the freeze-dried potato. Similarly, Wiktor et al. (2015), who studied influence of PEF on freezing and thawing of apple tissue, observed that the total freezing time and the total thawing time were reduced by, respectively, 3.5–17.2% and 71.5%. Similar results were reported by Ben Ammar et al. (2011)

Table 1 Examples of process conditions and effects of using PEF in food processing

Material	PEF parameters	Effect of PEF	References
Drying			
Basil (*Ocimum basilicum* L.) leaves	65 pulses of 650 V/cm, 150 µs pulse width, 760 µs between pulses	Drying times reduced 57% for air drying, 33% for vacuum drying and 25% for freeze drying	Telfser and Gómez Galindo (2019)
Parsnip and carrot	20 µs, 50 Hz, 0.9 kV/cm, after 1000 pulses	Drying time reduced to 28% at 70 °C and to 21% at 60 °C, compared to the untreated samples	Alam et al. (2018)
Carrot	Pulse number 10, 50 and 100; 1,85 and 5 kV/cm; 5,63, 8 and 80 kJ/kg	Drying time reduced up to 8.2%. Decrease of sample lightness up to 25.3%	Wiktor et al. (2016)
Potato tissue	300–400 V/cm	Decreasing the drying temperature approximately on 20°	Lebovka et al. (2007)
Extraction			
Citrus fruits and peel (orange, pomelo and lemon)	3 kV/cm—fruits 10 kV/cm—peel	Increased yield of juice by 25% for oranges, 37% for pomelos and 59% for lemon, improved extraction of polyphenols to 50%	El Kantar et al. (2018)
Fruit juice with the addition of stevia	30 kV/cm for 230 µs	The retention of ascorbic acid increased by over 74%. The enhancement of anthocyanins and carotenoids extraction	Carbonell-Capella et al. (2017)
	40 kV/cm for 230 µs	The highest content of hydroxymethylfurfural	Carbonell-Capella et al. (2017)
	21 kV/cm 300 µs with 2.5% stevia	The highest content of bioactive compounds and sweetening properties obtained with minimal color changes	Carbonell-Capella et al. (2017)
Blueberry fruits (*Vaccinium myrtillus* L.)	1,3 and 5 kV/cm, 10 kJ/kg	Increasing the juice yield (+ 28%) compared to the untreated sample. The juice obtained had a significantly higher total phenolic content (+ 43%), total anthocyanin content (+ 60%) and antioxidant activity (+ 31%)	Bobinaitė et al. (2015)
Freezing			
Baby spinach leaves	Two trains of bipolar, rectangular pulses with amplitude of 350 V, with 10 s interval between trains. Each train consisted of 500 pulses of 200 µs pulse width and 1600 µs of space between the pulses (frequency 500 Hz)	Improved freezing tolerance by applying vacuum impregnation and PEF in the presence of cryoprotectants	Demir et al. (2018)
Apple tissue	800 V/cm, pulse duration 1000 µs, time interval 100 ms, 10 pulses	Acceleration of cooling processes; good preservation of the macro-shape, inhibition of shrinking, development of large pores in the electroporated tissue	Parniakov et al. (2016a)
Beef muscle	1,4 kV/cm, 20 µs, 50 Hz, 250 kJ/kg (combined with freezing and thawing)	Microstructural changes in meat tissue, improved tenderness and purge loss	Faridnia et al. (2015b)
Preservation			
Fresh berries	2 kV/cm, pulse width 1 µs and 100 pulses per second for 2, 4 and 6 min + disinfectant solution (60 ppm peracetic acid [PAA])	The reduction of *E. coli* and *Listeria innocua* without changing the color and appearance of blueberries The softening of the berry structure Conc. of anthocyanins and phenolic compounds increased by 10 and 25%, respectively	Jin et al. (2017)
Peptides isolated from pine nuts	1800 Hz, 15 kV/cm	No changes of the amino acid sequence	Lin et al. (2017)

Table 1 continued

Material	PEF parameters	Effect of PEF	References
Milk	25.7 kV/cm for 34 μs after heating to 55 °C and maintained for 24 s and heat treatment at 63 °C for 30 min or at 73 °C for 15 min	Inactivation of alkaline phosphatase. Reduced xanthine (30%) and plasmin oxidase (7%) activity	Sharma et al. (2017)

and Al-Sayed et al. (2018). The authors concluded that electroporation of multicellular tissues led to better connections between intra- and extracellular content allowing increased probability of ice nucleation and faster ice propagation after freezing and correspondingly shortening the freezing time.

Food preservation

Food deterioration may be caused by several factors such as microorganisms development and activity of endogenous enzymes. The PEF technology, compared to the traditional pasteurization method, not only inactivates pathogenic microorganisms but also enzymes in some extent, minimizes the loss of the original taste, color, texture, nutrients and other thermolabile compounds found in food (Syed et al. 2017). For this reason, it is a promising supplement or substitute for traditional thermal pasteurization. PEF can be successfully used for liquid products with low viscosity and electrical conductivity, e.g. milk and juices.

Microbial inactivation

Milk and dairy products are processed using various thermal methods to make them safe for human consumption. Incorrect pasteurization of milk causes spoilage of the product and formation of pathogenic bacteria such as *Escherichia coli*, *Listeria* spp. and *Pseudomonas*. The treatment in which high temperatures are used causes nutrient losses (Ercolini et al. 2009). Pulsed electric field not only inactivates bacteria at low temperatures, but also affects minimally the nutritional and sensory properties of the food product. PEF causes inactivation of Gram-negative and Gram-positive bacteria in a whole milk already at 50 °C (Sharma et al. 2014). Milk that has been thermally preserved can be microbiologically stable for 21 days when stored at 4 °C. However, heat causes unfavorable effects such as: damage to the creaming properties, non-enzymatic browning, degradation of lactose, denaturation of whey proteins (Fox et al. 2015). PEF technology can be used synergistically with heat, antimicrobial agents, membrane filtration and ultraviolet radiation in order to increase the effectiveness of bacterial inactivation and prolonging the period of consumption. In one of the studies of Sharma et al. (2017), milk samples were subjected to PEF with the following parameters: 25.7 kV/cm for 34 μs after heating to 55 °C and maintained for 24 s and heat treatment at 63 °C for 30 min or at 73 °C for 15 min. Inactivation of alkaline phosphatase was comparable in all samples. The PEF-treated sample initially exhibited reduced xanthine (30%) and plasmin oxidase (7%) activity, however, after 21 days of refrigeration storage, these parameters were similar to the milk sample not processed at all. During storage in all milk samples, lipolytic activity increased and the pH level dropped. Hemar et al. (2011) reported that PEF has no impact on whey proteins and milk pH, but it can affect the viscosity and particle size when milk is treated with high field strengths.

Jin et al. (2017) studied how PEF affects the native microflora and the population of *E. coli Listeria innocua*, which have been artificially grafted on blueberries. The combination of PEF and PAA (60 ppm peracetic acid) resulted in the reduction of *E. coli* and *Listeria innocua*, but it did not change the color and appearance of blueberries. The only disadvantage of the process was the softening of the berry structure. Anthocyanins and phenolic compounds increased by 10 and 25%, respectively. Palgan et al. (2012) combined PEF and manothermosonication (MTS) to reduce *Listeria innocua* in a milk based smoothie. The study showed that the application of MTS followed by PEF was the most effective in inactivating *L. innocua* causing a mean reduction of 5.6 log cfu/ml.

Spore inactivation

PEF processing seems to have no effect on endospores although some publications describe a certain level of spore inactivation achieved if the necessary harsh conditions are applied. Spores present a higher resistance to PEF than vegetative cells due to their small sizes, low permeability, dehydration and mineralization (Setlow 1995). Therefore, at present, PEF treatment alone can be applied for pasteurization but not for sterilization purposes.

However, combined application of PEF with other methods e.g. thermal treatment can lead to successful inactivation of endospores. For instance, Siemer et al. (2014) reported a 3 log cycles inactivation of *B. subtilis* spores under the following process conditions: electric field strength of 9 kV/cm, inlet temperature of 80 °C, the addition of 10% sugar to medium. Similarly, Reineke et al.(2015) achieved a 4.67 $\log_{10}$ inactivation of *B. subtilis* spores in saline water when they applied the process conditions: 70 °C with a flow rate of 5 l/h, a frequency of 150 Hz, an energy input of 226.5 kJ/kg.

Enzyme inactivation

Enzymes are less sensitive to the action of PEF than microbes so more intense PEF treatments are required for their inactivation (Ho et al. 1997), but the mechanism of this phenomenon is still not well understood. Probably both electrochemical and thermal effects, which are associated with PEF, cause the changes in the structure and conformation of enzymes leading to their inactivation (Terefe et al. 2013). In the case of grape juice, which is susceptible to the action of many enzymes, PEF did not significantly affect its physicochemical and sensory characteristics but it reduced the activity of polyphenyl oxidase and peroxidase. The duration of PEF action, its intensity and frequency had a significant effect on the relative activity of selected enzymes, which was abolished along with the increase of the above-mentioned parameters (Marselle´s-Fontanet and Martin-Belloso 2007).

Extraction of bioactive compounds

Extraction is one of the most commonly used processes in the industry to obtain valuable compounds and usually it involves chemical and/or thermal treatment of a sample. Numerous studies report that application of pulsed electric field for extraction can enhance its efficiency, reduce the extraction time and minimize any damage to the extracted nutrients. PEF has been used to improve the extraction of intracellular compounds from fruits and vegetables. Luengo et al. (2013) reported that amount of polyphenols extracted from tomatoes and grapes increased after treatment with PEF. The use of this technique enhanced also extraction of polyphenols from borage (*Borago officinalis* L.) leaves and increases their antioxidant activity. In addition, it also reduced the extraction time, and the increase in pulse intensity was proportional to the amount of polyphenols extracted, as well as to their antioxidant properties (Segovia et al. 2014). Soliva-Fortuny et al. (2017) studied the effect of PEF on the content of phenols, flavonoids and flavan-3-ol as well as on the antioxidant capacity of apples stored at different temperatures (4 and 22 °C) for 48 h. The maximum increase in the total phenol content (13%) and flavone-3-ol (92%) was observed in apple treated with the mildest electric field parameters. The antioxidant activity was also higher in apples subjected to PEF (by 43%) in relation to the untreated samples. Liu et al. (2018a, b) investigated the effect of PEF on the extraction of water-soluble phenolic compounds from onion as well as the antioxidant activity of the extracts. Results indicated that the yield of water-soluble phenolic and flavonoid compounds extracted from onion significantly increased after PEF treatment following water extraction, by 2.2 and 2.7 times, respectively, in comparison to control. The authors noted that the antioxidant activity of extracts increased with the increase in electric field intensity and treatment time. El Kantar et al. (2018) investigated the effect of PEF on citrus fruits (orange, pomelo and lemon). Fruits and peel were treated with a pulsed electric field at a field voltage of 3 kV/cm and 10 kV/cm, respectively. PEF processing increased the yield of juice by 25% for oranges, 37% for pomelos and 59% for lemon, and improved the extraction of polyphenols to 50%.

PEF is an ideal method used to enhance the extraction process of various intracellular compounds, e.g. sugar from sugar beet (Lopez et al. 2009), phytosterols from maize germs (Guderjan et al. 2005). One of the benefits of using PEF is also obtaining high purity of fruit juices (Lebovka et al. 2003). However, the use of too high intensity can lead to destruction of the cell membrane, cell turgor and may have an adverse effect on the viscosity and elasticity of plant tissue (Lebovka et al. 2004). The ability of PEF to inactivate microorganisms and induce the permeabilization of eukaryotic cells without a significant increase in the temperature of the product can be used in the process of wine production to improve its quality. The low energy consumption and short processing time required to permeabilize grape skin cells are the key advantages of using PEF in obtaining wines with a high content of phenolic compounds. The high concentration of polyphenols helps in stabilizing the color and improves the quality of the wine during the aging process (Boulton 2001). Phenolic compounds also have pro-health activities (e.g. antioxidant and pro-inflammatory properties). PEF does not affect the change in the taste, color or nutritional value of grape must and wine. It also facilitates the growth of active dry wine yeast that is added to the grape must to provide a faster fermentation process. PEF also reduces the amount of SO_2, which spoils the quality of wine (Puertolas et al. 2010).

The fruit juice with the addition of stevia was processed by means of PEF to study the effect of this technology on bioactive compounds and steviol glycosides. PEF treatment resulted in the retention of ascorbic acid by over 74%, the enhancement of anthocyanins and carotenoids extraction. The best results were obtained at 30 kV/cm for 230 µs. At

the highest voltage of 40 kV, the highest hydroxymethylfurfural content was found. With PEF carried out at 21 kV/cm during 300 µs with 2.5% stevia, the highest content of bioactive compounds and sweetening properties was obtained with minimal color changes (Carbonell-Capella et al. 2017).

Lin et al. (2017) investigated the mechanism of improving the antioxidant properties of peptides isolated from pine nuts using PEF. Radical inhibition of DPPH and cellular antioxidant activity (CAA) were used to assess the antioxidant activity of peptides. The structure of electroporated peptides was analyzed by medium-infrared spectrophotometry (MIR) and circular dichroism (CD). The capture of DPPH radicals increased significantly (89.10% ± 0.20% to 93.22% ± 0.09%) under PEF treatment conditions. The pulse frequency was 1800 Hz and the electric field voltage was 15 kV/cm. PEF did not change the amino acid sequence of Gln-Cys-His-Lys-Pro, Gln-Cys-His-Gln-Pro, Lys-Cys-His-Gln-Pro.

Starch modification

Pulsed electric field can be used for modification of potato, corn, wheat, waxy rice, and cassava starches (Han et al. 2009; Hong et al. 2018; Li et al. 2019; Zeng et al. 2016). The researchers observed re-arrangement and destruction of starch molecules as well as a decrease in gelatinization properties, viscosity and crystallinity along with the increase in field strength (1.25–5 kV/cm and 30–50 kV/cm). Application of PEF affected starch digestibility increasing level of rapidly digestible starch in potato, wheat and pea starches (PEF intensity of 2.86, 4.29, 5.71, 7.14, and 8.57 kV/cm, 600 Hz of pulse frequency, 6 µs of pulse width) (Li et al. 2019) and in waxy rice starch (30, 40 and 50 kV/cm) (Zeng et al. 2016). Hong et al. (2018) reported that such starch modification methods like acetylation can be significantly enhanced by PEF treatment (PEF parameters: pulse frequency of 1000 Hz; field intensity of 1.25, 2.50, 3.75, and 5.00 kV/cm; pulse duration time of 40 µs). The use of PEF to support the starch modification methods can enhance the process's efficiency, reduce reaction time and save reagents.

Waste valorisation in the food industry

Food industry generates huge quantities of by-products and wastes, which are problematic because their disposal is associated with environmental and health related issues. On the other hand, they still can be rich sources of natural bioactive compounds, especially in the fruit and vegetable industry. Recently, much attention has been paid to the use of emerging technologies, including PEF, for the recovery of these compounds. For instance, Ghosh et al. (2019) proposed combining PEF with mechanical pressing for extraction of functional molecules from the waste chicken breast muscle. Andreou et al. (2020) applied PEF in tomato processing to enhance valorization of tomato waste. They noticed the increase in extraction yield of carotenoid up to 56.4%. Lycopene extraction also increased (from 9.84 mg lycopene/100 g to 14.31 mg/100 g tomato residue) for a PEF treatment at 1.0 kV/cm for 7.5 ms. The concentration of extracted total phenolic compounds doubled when tomato waste was treated with a 2 kV/cm and 700 pulses. Other exemplary studies concern the application of PEF for enhancing the extraction of polyphenols from lemon peel residues (Peiró et al. 2019), potato peels (Frontuto et al. 2019), mango and papaya by-products (Parniakov et al. 2016b).

Impact of PEF on nutrients and bioactive compounds

Studies published recently have been performed mainly in plant-based products, especially juices. They have shown that PEF treatment can be regarded as safe for such bioactive compounds as vitamins, carothenoids and polyphenols. No significant changes in content of vitamin C were reported for apple juice (200, 300, and 400 pulses, electric field strength 30 kV/cm) (Dziadek et al. 2019), pineapple juice (20, 30 and 40 kV and frequency 10, 20, 30 and 40 kHz) (Indriani et al. 2019) or blueburry juice (350 V) (Zhu et al. 2019). It seems that PEF has no impact on the bioaccessibility of this vitamin (Rodríguez-Roque et al. 2015). Salvia-Trujillo et al. (2011), who studied the effect of PEF processing on content of B vitamins in a beverage containing fruit juices (orange, kiwi, mango, and pineapple) and whole and skim milk, found that niacin and thiamin contents in the fruit beverages were not affected by PEF treatment (electric field strength of 35 kV/cm for 1800 µs, a pulse frequency of 200 Hz, and 4 µs bipolar pulses). The use of PEF for pasteurization has the advantage over heat treatment to preserve bioactive compounds. For example, higher concentrations of phenolic acids and flavonoids were observed in PEF-treated tomato juice and orange juice as compared to the conventional thermally treated samples (Odriozola-Serrano et al. 2008; Agcam et al. 2014). PEF pasteurisation of milk maintains its nutritional value. Studies showed that there was no significant effect of pasteurization in a continuous PEF bench scale system (35 kV/cm field strength with 64 pulses of bipolar square wave for 188 µs) on proteins and total solids in milk (Michalac et al. 2003). Similarly, no changes were detected in the retention of thiamine, riboflavin, retinol, cholecalciferol and α-tocopherol in skim milk and fresh bovine whole milk subjected to PEF treatment at

18.3–27.1 kV/cm, 400 ms, at 50–90 °C (Bendicho et al. 2002) or 15–35 kV/cm, 12.5–75 ms at 30 °C (Riener et al., 2009). However, whole milk treated with PEF (20–35 kV/cm, 24–60 ms, 20–40 °C) demonstrated a small reduction of the fat content (Bermúdez-Aguirre et al. 2011). More studies are needed to find out how PEF affects milk protein, as the results published so far are inconsistent. No negative effects on the quality and functionality of oils have been shown in the studies concerning the use of PEF (1.8 kV/cm, 1.6 kJ/kg) for enhancing oil extraction form olives (Andreou et al. 2017). In the case of meat and fish products the number of studies is still too small to draw far-reaching conclusions on the impact of PEF on their nutritional value.

The potential use of PEF in production of food with increased nutritional value

Pulsed electric fields has been be applied for enrichment of microorganisms in ions essential for proper functioning of human organism (Table 2). Cell biomass prepared in this way may potentially be used for production of functional food. Yeasts are known for their ability to accumulate metal ions from aqueous solutions, e.g. by adsorption and absorption or metabolism (Cha and Cho 2009). Bioaccumulation of metal ions by *Saccharomyces cerevisiae* strains takes place in two stages. In the first stage, biosorption or "passive capture" takes place. It is independent of yeast metabolism and associated with the accumulation of cations on the outer surface of the cell wall. The metal ions are then adsorbed to the anionic sites. The second stage called bioaccumulation or "active capture" is already dependent on the cell metabolism and involves the penetration of metal ions into the cell by means of specific membrane transporters. Metal ions accumulate in vacuoles (MacDiarmid et al. 2002). This mechanism of cation binding may lead to the formation of organic linkages called "bioplexes". It has been shown that protein and mineral complexes (metaloproteins or bioplexes) are very well assimilated by human organism (De Nicola et al. 2007). The use of yeast or bacteria as a carrier of bioplexes can help to enrich diets with deficient elements such as magnesium, zinc, calcium or selenium. The studies of Pankiewicz and Jamroz (2013), Pankiewicz et al. (2014) showed that yeast cells treated with PEF can accumulate magnesium, zinc and calcium more efficiently due to the phenomenon of electroporation. The authors reported that bioaccumulation of magnesium was 1.5 times higher, zinc—two times higher and calcium even 6 times higher when compared to the culture not treated with PEF. Observations of the PEF-treated yeast cells using laser confocal microscopy revealed that zinc ions are dispersed mainly in cell organelles, and magnesium ions—in the cell wall (Pankiewicz et al. 2015).

Application of PEF can also enhance bioaccumulation of magnesium ions in *Lactobacillus rhamnosus* B 442, *Lactobacillus rhamnosus* 1937 and *Lactococcus lactis* JBB 500 cells, which can then be used for production of ice cream. The addition of bacteria enriched with Mg^{2+} did not affect the physicochemical characteristics (freezing, fusibility, hardness) of the ice cream and did not change the color of the samples. The higher total number of microorganisms was noted in the ice cream than in the starter cultures, however, the viability of these bacteria was lower than in the control samples (Góral et al. 2018). Góral et al. (2019a, b) used PEF for enhancing bioaccumulation of calcium and zinc in the cells of *Lactobacillus rhamnosus* B 442. The highest bioaccumulation of zinc was observed when the following PEF parameters were applied: field strength 3 kV/cm, pulse width 20 µs and electroporation time of 20 min. The optimal PEF parameters for calcium accumulation were as follows: field strength 3.0 kV/cm, exposure time 10 min, and pulse width 75 µs. Bioaccumulation of Zn^{2+} and Ca^{2+} was higher than in the control sample (with the addition of zinc and without PEF treatment) by, respectively, 164% and 300%. *Lactobacillus rhamnosus* B 442 cells enriched with zinc ions were used for the production of two types of ice cream: unfermented and fermented (Pankiewicz et al. 2019). Also in the case of Se^{2+}, the application of pulsed electric fields improves accumulation of this element in yeast cells up to 68% (Pankiewicz et al. 2017).

Results of some studies indicate that the use of PEF during juice production may result in higher content of vitamins and polyphenolic compounds compared to those obtained by traditional technology. Odriozola-Serrano et al. (2008) used PEF (electric field strength of 35 kV/cm, field frequency in the range of 50–250 Hz and pulse width from 1 to 7 µs) to obtain strawberry juice with a higher nutritional value in terms of vitamin C, anthocyanins and antioxidants contents. They observed 98% retention of vitamin C, from 83 to 102% retention of anthocyanins, whereas retention of the antioxidants ranged from 75 to 100%. Maximum retention was obtained when bipolar impulses were applied at field strength of 35 kV/cm, pulse width of 1 µs and frequency of 250 Hz. Cortés et al. (2006) noticed that content of vitamin A in orange juice treated with PEF was higher by 8.1% than in the pasteurized juice. Salvia-Trujillo et al. (2011) showed that beverages containing milk and fruit juice (kiwi, mangoes, oranges and pineapples) treated with high intensity PEF had higher vitamin B2 content than those which were treated thermally. Agcam et al. (2014) in comparative studies of orange juice treated with PEF and thermal pasteurization found that flavonoids and phenolic acids in PEF-treated

Table 2 An overview of studies on the application of PEF for increasing biosorption of the selected elements by microorganisms

Microorganism	Nutrient	Conditions of PEF treatment	Effects	References
Saccharomyces cerevisiae	Magnesium ions	15-min exposure of the 20-h grown culture to PEF of the 2000 V and pulse width 20 μs; magnesium concentration of 100 μg/mL	Accumulation of magnesium in the yeast biomass reached maximum 3.98 mg/g dm	Pankiewicz and.Jamroz (2010)
Saccharomyces cerevisiae	Zinc ions	15 min exposure of the 20 h grown culture to PEFs of 1500 V and 10 μs pulse width; 100 μg Zn/mL medium	Accumulation of zinc in the yeast biomass reached a maximum of 15.57 mg/g d.m (63% higher than in the control)	Pankiewicz and Jamroz (2011)
Saccharomyces cerevisiae	Calcium ions	20 min exposure of the 20 h grown culture to PEF of the 5.0 kV/cm and 20 μs pulse width; calcium concentration 100 μg/mL medium	Bioaccumulation of calcium in the yeast biomass reached maximum 2.98 mg/g d.m. It constituted 30% of the total calcium in the medium	Pankiewicz and Jamroz (2013)
Saccharomyces cerevisiae	Magnesium and zinc ions	15 min exposure time, culture grown for 20 h field strength of 5.0 kV/cm, pulse width of 20 μs; concentration of 100 μg Mg^{2+}/mL and 150 μg Zn^{2+}/mL medium	Bioccumulation of magnesium and zinc reached maximum levels of 2.85 and 11.41 mg/g d.m., respectively. Optimization of ion pair concentration and PEF parameters caused a 1.5 or twofold increase of Mg and Zn accumulation, respectively	Pankiewicz et al. (2014)
Saccharomyces cerevisiae	Selenium and zinc ions (simultaneously)	Electric field strength of 3 kV/cm and pulse width of 10 μs, treatment of 20-h culture for 10 min; ion con. − 100 μg Se/mL and 150 μg Zn/mL medium	Increase of ions accumulation by 65% for selenium (43.07 mg/g d.m.) and 100% for zinc (14.48 mg/g d.m.)	Pankiewicz et al. (2017)
Lactobacillus rhamnosus B 442	Magnesium ions	5 min exposure of the 20 h grown culture to PEF of the 2.0 kV/cm and 20 μs pulse width at conc. 400 μg Mg^{2+}/mL medium	PEF caused an increase of magnesium concentration in the cells by 220% in comparison to the control not treated with PEF, accumulation of magnesium in the biomass reached maximum 4.28 mg/g d.m.	Góral, Pankiewicz (2017)
Lactobacillus rhamnosus B 442, *Lactobacillus rhamnosus* 1937, and *Lactococcus* lactis JBB 500	Magnesium ions	5 min at pulse width 20 μs, electric field strength 2.0 kV/cm, at the field frequency of 1 Hz; ion conc. of 400 μg Mg^{2+}/mL medium	The highest concentration—4.28 mg Mg^{2+}/g d.m., was obtained for *L. rhamnosus B* 442. The strains *L. rhamnosus* 1937 and *L. lactis* JBB 500 accumulated, respectively, 1.97 mg Mg^{2+}/g d.m. and 1.86 mg Mg^{2+}/g d.m.	Góral et al. (2018)
Lactobacillus rhamnosus B 442	Zinc ions	Field strength of 3.0 kV/cm, pulse width of 20 μs, electroporation time of 15 min after 20 h of culturing and at zinc conc. of 500 μg/mL medium	Bioaccumulation of zinc increased by 164% compared to the control (no PEF). The maximum content of zinc ions from cells was 2.85 mg Zn^{2+}/g d.m. PEF did not reduce bacterial viability or biomass	Góral et al. (2019a, b)

one were more stable than in juice treated with the thermal pasteurization. PEF also allows the preservation of the initial content of fatty acids and amino acids in the product. Zulueta et al. (2007) did not notice a decrease in the content of saturated, monounsaturated or polyunsaturated fatty acids in orange juice-milk beverage fortified with n − 3 fatty acids and oleic acid processed by high-pulsed electric field. Morales-De La Peña et al. (2012) noted that the content of free amino acids in a fruit juice-soy milk beverage treated with high intensity pulsed electric field and stored at 4 °C was higher than in the beverages thermally treated. On the other hand, the content of histidine, tyrosine, methionine and leucine was lower in the beverages subjected to thermal pasteurization.

PEF can also be used to support the formation of an iron-glycine complex which is stable and has good bioavailability. Zhang et al. (2017) obtained the highest yield of the Fe-glycine complex (81.2%) and the highest iron chelation capacity (107.13 mg/L), using PEF with an electric field strength of 4 kV/cm, frequency of 1 kHz and

pulse width of 40 μs for 15 min. The yield value obtained was higher than in the case of the complex formed by thermal treatment (30 min, 60 °C). Based on the results obtained, the authors concluded that PEF can be used in industry to form metal ion complexes with protein amino acids.

Pharmacological effects (e.g. anti-inflammatory, anti-cancer, antioxidant) of C-phycocyanin (C-PC) derived from *Spirulina platensis*, caused this compound to have potential applications in the production of functional foods (Liu et al. 2016). Phycocyanin is a pigment-protein complex with a blue color, which is widely used as a natural food color in the food industry (Taufiqurrahmi et al. 2017). Martinez et al. (2017) used PEF to enhance extraction of C-phycocyanin from *A. platensis*. The purity of the extract obtained from PEF-treated cells was much higher than in the case of other techniques that consisted of complete destruction of the cell.

The development of technology allows the introduction of "novel food" to trade. An example of such food are proteins obtained from microalgae *Ulva* sp. Protein extraction from these microorganisms is possible due to the use of chemical substances, however this method has serious consequences, therefore PEF combined with osmotic shock and mechanical press was used as an alternative extraction method. Subsequently, the extracted proteins were identified and a specific allergen was assigned to them. Extracts that were obtained with PEF contained only one food allergen—superoxide dismutase (SOD), however, more research is needed on the allergenicity of proteins extracted from macroalgae to assess the risk for human consumption (Polokovsky et al. 2019).

Conclusion

The present review discussed the selected current and potential applications of PEF in food industry. Development of new technologies in food processing is forced, among others, by the growing interest of consumers in fresh-like products of high nutritional value, and the demand for food produced with the use of environmentally friendly methods. PEF is a method that uses electric waves with high voltage amplitude. Short electrical impulses (from microseconds to milliseconds each) of high voltage (typically 10–80 kV/cm) are supplied to the product placed between the electrodes in the chamber. This technology can be used alone or in combination with other methods to obtain products in more energy efficient (e.g. by lowering temperature and time of extraction) and environmentally friendly way. PEF can be applied for pasteurization, enhancement of such processes as drying, freezing, or extraction, but can also support development of functional food containing e.g. easily absorbed ions of elements essential for proper functioning of the human body.

Research of pulsed electric fields technology is carried out around the world. Although this technology has been investigated extensively and there already are commercial PEF systems working in different countries, the majority of the obtained results still refer to the experiments carried out at laboratory scale.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Pulsed Electric Field (PEF) Enhances Iron Uptake by the Yeast *Saccharomyces cerevisiae*

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Abstract: The aim of the study was to investigate the influence of a pulsed electric field (PEF) on the level of iron ion accumulation in *Saccharomyces cerevisiae* cells and to select PEF conditions optimal for the highest uptake of this element. Iron ions were accumulated most efficiently when their source was iron (III) nitrate. When the following conditions of PEF treatment were used: voltage 1500 V, pulse width 10 µs, treatment time 20 min, and a number of pulses 1200, accumulation of iron ions in the cells from a 20 h-culture reached a maximum value of 48.01 mg/g dry mass. Application of the optimal PEF conditions thus increased iron accumulation in cells by 157% as compared to the sample enriched with iron without PEF. The second derivative of the FTIR spectra of iron-loaded and -unloaded yeast cells allowed us to determine the functional groups which may be involved in metal ion binding. The exposure of cells to PEF treatment only slightly influenced the biomass and cell viability. However, iron-enriched yeast (both with or without PEF) showed lower fermentative activity than a control sample. Thus obtained yeast biomass containing a high amount of incorporated iron may serve as an alternative to pharmacological supplementation in the state of iron deficiency.

Keywords: iron accumulation; yeast; iron deficiency; PEF

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1. Introduction

Anemia affects about one-third of the world's population; half of these cases are caused by iron deficiency. This is a serious and global public health problem that affects maternal and child mortality and their overall physical function. Children 0–5 years old, women of childbearing age and pregnancy are particularly at risk [1].

Iron is essential for numerous biological functions, including respiration, energy production, DNA synthesis and cell proliferation [2,3]. A sufficient supply of iron is necessary for the correct functioning of many biochemical processes, including electron transfer reactions, gene regulation, oxygen binding and transport, and regulation of cell growth and differentiation [4]. Iron deficiency is associated with chronic kidney disease, chronic heart failure, cancer and inflammatory bowel disease [5].

In addition to seeking and treating the cause of iron deficiency, treatment strategies include prevention, through iron supplementation and food enrichment [6]. Iron supplementation is necessary for high-risk groups (e.g., pregnant women). For oral supplementation, iron salts (ferrous sulfate and ferrous gluconate) are preferred because of their low cost and high bioavailability. Although iron absorption is increased when given on an empty stomach, nausea and epigastric pain may occur. Administration of iron during a meal reduces its absorption by approximately two-thirds [7]. Side effects of iron supplementation can be reduced by introducing iron-enriched foods into the diet [8].

Food products such as wheat flour, milk and infant formulas have been long used for iron fortification. Many countries have implemented programs of enrichment staple foods in iron, but they have disadvantages as some members of the population may not consume these products or have no regular access to these foods. Enriching food with iron is more difficult than with other nutrients such as iodine in salt and vitamin A in frying oil, because iron affects the sensory characteristics, changing the taste and color of the product. It can also cause fat oxidation [9]. To prevent these adverse changes, the microencapsulation process is used [10]. One of the encapsulation methods is the accumulation of minerals in the yeast cells of *S. cerevisiae* [11–14]. In the study of Kyyaly et al. [15] it was demonstrated that feeding anemic rats with iron-enriched yeast is more efficient than inorganic treatment in recovery from iron deficiency. In this case iron bioavailability from enriched yeast was higher than from ferrous sulfate heptahydrate.

Yeast is a single-cell organism that is used in brewing (beer and wine production), ethanol production, baking, and the production of recombinant proteins and biopharmaceuticals. Although there are many yeast species, *Saccharomyces* is by far the most commercially available [16]. Easily-grown and readily available yeasts such as baker's or brewer's strains of *S. cerevisiae* are excellent natural sources of essential metals such as K, Mg, Ca, Fe, Mn, and Zn and this yeast can be further enriched with other inorganic micronutrients (e.g., selenium) [17]. Metal accumulation in yeast involves a combination of extracellular accumulation and transport mechanisms. The first stage called biosorption is a reversible stage of accumulation. The second stage, usually referred to as active transport, is slower intracellular bioaccumulation, which is often irreversible and is associated with cellular metabolic activity [18]. The cell membrane is a barrier between the cell and its external environment. However, under special circumstances, the membrane barrier and transport selectivity can be partially lost, and molecules that do not pass through the intact cell membrane can get into the cell's cytoplasm. This phenomenon occurs when cells are exposed to a pulsed electric field (PEF) [19].

The pulsed electric field (PEF) technique can be used as a non-thermal food preservation method that involves the use of short electrical pulses to reduce the microbial population while having minimal detrimental effects on food quality [20]. This process is also used to assist drying and can also cause stress reactions in plant systems or cell cultures [21]. Previous research has shown that this technique can be used for enriching *S. cerevisiae* yeast with metal ions [11,22,23]. Iron enriched yeast biomass can potentially be an additional source of this element in diet, especially for vegans and vegetarians.

The aim of the study was to investigate the influence of a pulsed electric field on the level of iron accumulation in *S. cerevisiae* cells. The ATR FTIR analysis was applied to determine the functional groups in yeast cells that could be involved in iron ions' binding and demonstrate the effectiveness of PEF in enhancing the uptake of iron.

2. Results and Discussion

2.1. Selection of Iron Salt

In the first stage of the experiment we selected the iron salt for which the highest accumulation of this element in yeast was observed. Cells derived from the culture not supplemented with iron and not exposed to PEF contained only 0.13 mg Fe/g dry mass and were used as control (C1). This level of iron accumulation is in agreement with that reported by Zachariadis et al. [24] for active dry yeast. In our experiments each iron salt was added to two cultures and one of them was additionally treated with PEF. Studies have shown that the greatest effect of PEF on iron accumulation in *S. cerevisiae* cells occurred when iron (III) nitrate was used as the source of iron ions (Figure 1). This may be related to the fact that iron (III) nitrate is derived from a strong acid and is likely to be in a dissociated form, thus providing free iron for binding to cells [25]. In the case of this salt, the iron concentration in yeast cells was almost 97 times higher in the PEF-treated sample than in the control sample with no iron salt added and not subjected to PEF (C2). For the remaining salts, no significant effect of PEF on iron accumulation was observed. Generally,

yeast accumulated the least amount of iron from iron citrate, which may be due to the change in pH of the medium from 5.0 to about 4.4. Lower pH may cause incomplete dissociation of iron citrate, which explains the lower intracellular iron content compared to other salts [26]. Additionally, some authors have observed a toxic effect of iron (III) citrate on yeast cells [27,28]. According to Paš et al. [28] this effect can be caused not only by iron, but also by the anionic part of this iron compound.

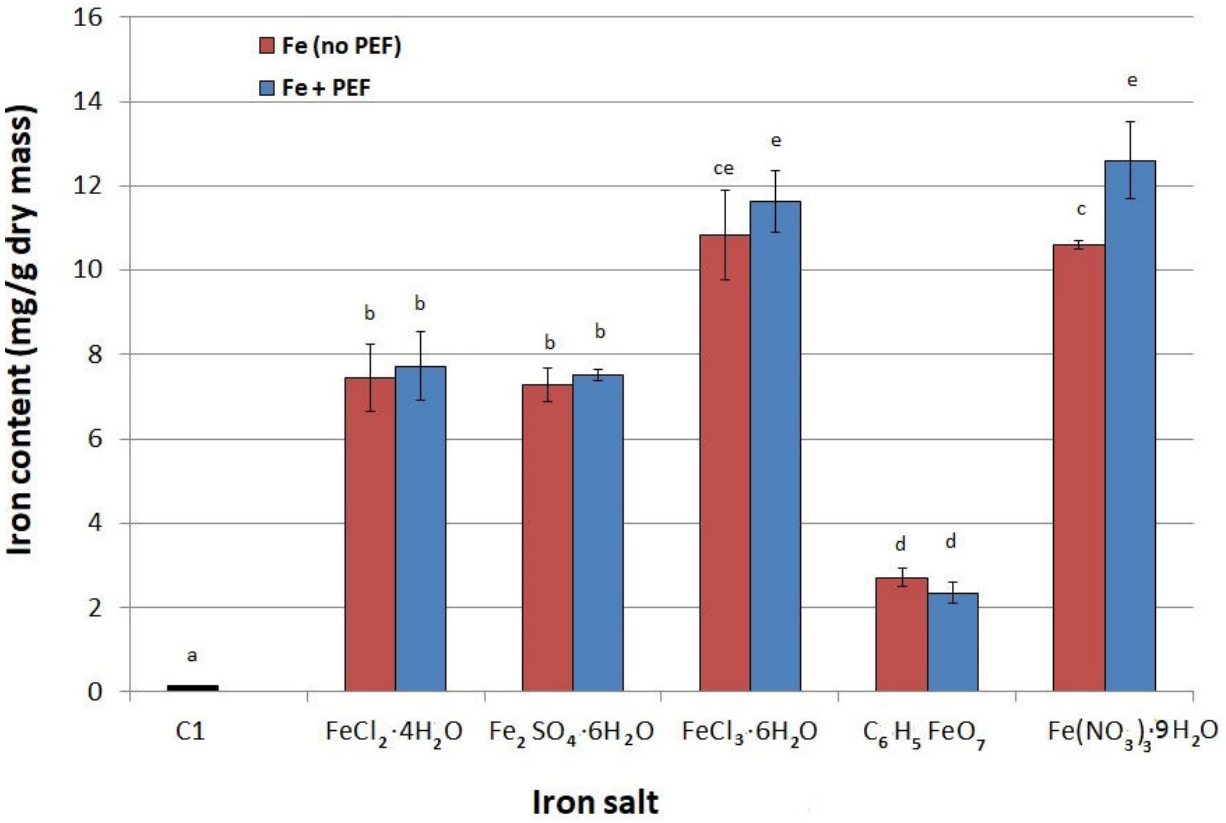

Figure 1. Effect of iron salt on iron accumulation in yeast cells: C1—control culture without iron ions added to the medium and PEF treatment; red bars—cultures not treated with PEF, blue bars—cultures treated with PEF (100 µg Fe/mL medium, voltage of 1500 V, pulse width of 10 µs, treatment time 10 min, number of pulses 600, after 20 h of cultivation). Each value is the mean ± standard deviation (n = 3). Bars with the same letter (a–e) are not significantly different ($p < 0.05$).

Our experiments showed that at low concentrations of iron ions in the culture medium (in the range of 50–100 µg/mL) PEF treatment had no effect on iron accumulation in yeast. Statistically significant changes were noted at 200 µg Fe^{3+}/mL, and then the difference in accumulation between cells supplemented with iron without PEF treatment and PEF-treated cells was the largest and amounted to 8.23 mg/g dry mass. We observed that iron uptake was reduced when concentration was higher than 200 µg Fe^{3+}/mL (Figure 2), so we adopted the concentration of 200 µg Fe^{3+}/mL as optimal for effective accumulation of this element in further experiments. High iron ions concentration in the medium may cause precipitation reactions, which may be due to the formation of iron hydroxides, their polymerization or the formation of poorly soluble phosphate iron [28]. Iron is an essential ingredient for yeast, but its high content in the culture medium can also be toxic. For this reason the uptake and utilization of iron in yeast cells is tightly regulated [29]. *S. cerevisiae* can grow in environments with both too little and too much iron [30]. A study by Philpot and Protchenko [25] indicates that when iron is restricted, cells will not only increase iron uptake, but also adjust their metabolism to use the available iron more efficiently.

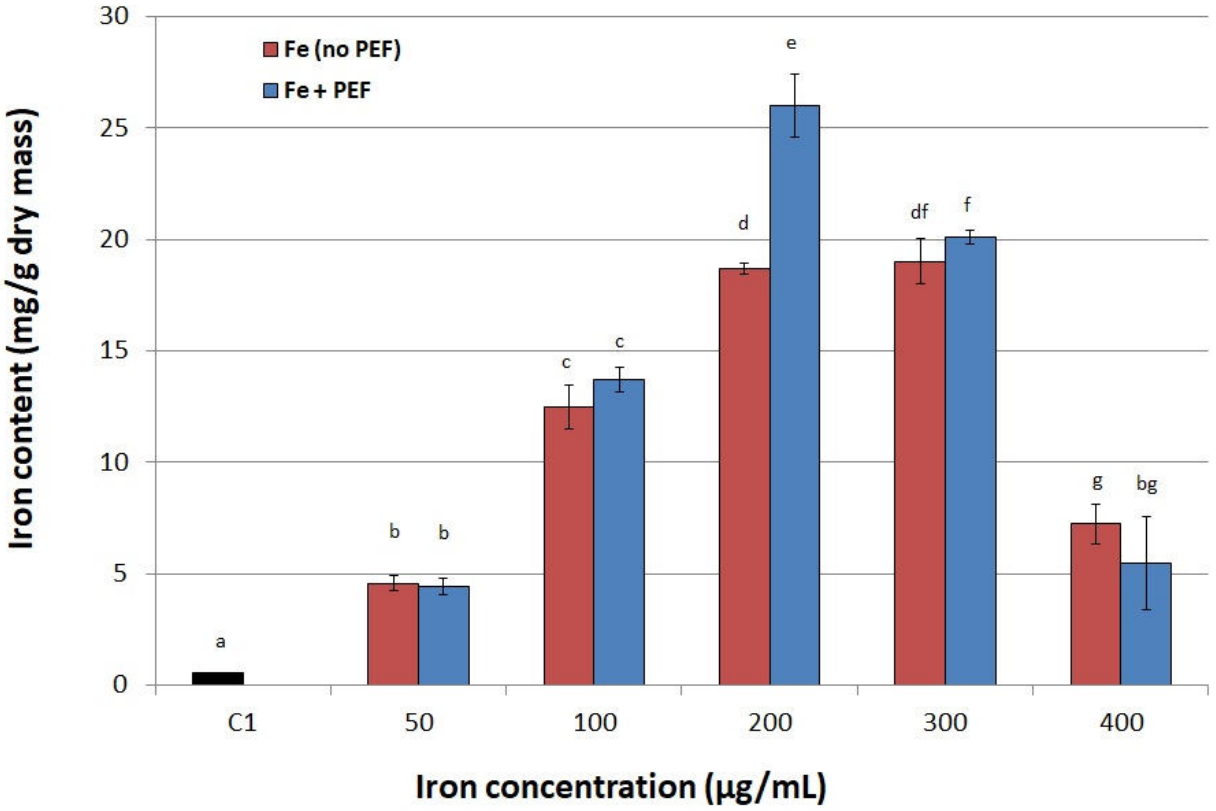

Figure 2. Effect of iron concentration on its accumulation in yeast cells. C1—control culture without iron ions added to the medium and PEF treatment; red bars—cultures not treated with PEF, blue bars—cultures treated with PEF (ferric nitrate, voltage of 1500 V, pulse width of 10 µs, treatment time 10 min, number of pulses 600, after 20 h of cultivation). Each value is the mean ± standard deviation (n = 3). Bars with the same letter (a–g) are not significantly different ($p < 0.05$).

2.2. Conditions of PEF Treatment

Figures 3–5 present the effect of PEF parameters on biomass production and cell viability, as well as on iron accumulation in cells. Only slight fluctuations in the number of inactivated cells in the entire range of tested values of voltage were observed. The highest fraction of dead cells in a culture (10%) was observed at 3000 V (Figure 3A). A significant drop in biomass production (from 0.87 to 0.78 g dry mass/100 mL) was noted only at voltages higher than 2000 V. In the case of pulse width, we observed a significant decrease in biomass in the range from 75 µs to 150 µs at 1500 V. The pulse width also influenced the viability of yeast cells (Figure 4A). At 10 µs it was the same as in the control not exposed to PEF (C2), however, for values higher than 50 µs, cell viability began to decline. But still we noted high values for both parameters—the share of dead cells did not exceed 10% and biomass was over 0.7 g dry mass/100 mL. Similarly, Stirke et al. [31] did not observe a significant impact of pulse durations of less than 100 µs on yeast cells' viability. Treatment time had little effect on biomass and cell viability (Figure 5A). Although the biomass production dropped significantly already after 5 min treatment, compared to the control cultures, this decrease was not sharp. After 20 min of treatment the biomass production was 15% lower than in the control sample not exposed to PEF, but the total number of inactivated cells was rather low (only 8%). Longer treatment resulted in a higher decrease in biomass and cells viability.

Temperature is also a critical parameter that influences the efficacy of PEF treatment [32]. In our experiments the temperature was continuously monitored and was between 24 and 26 °C. There were no drastic changes that could influence the conductivity of the medium or cell growth.

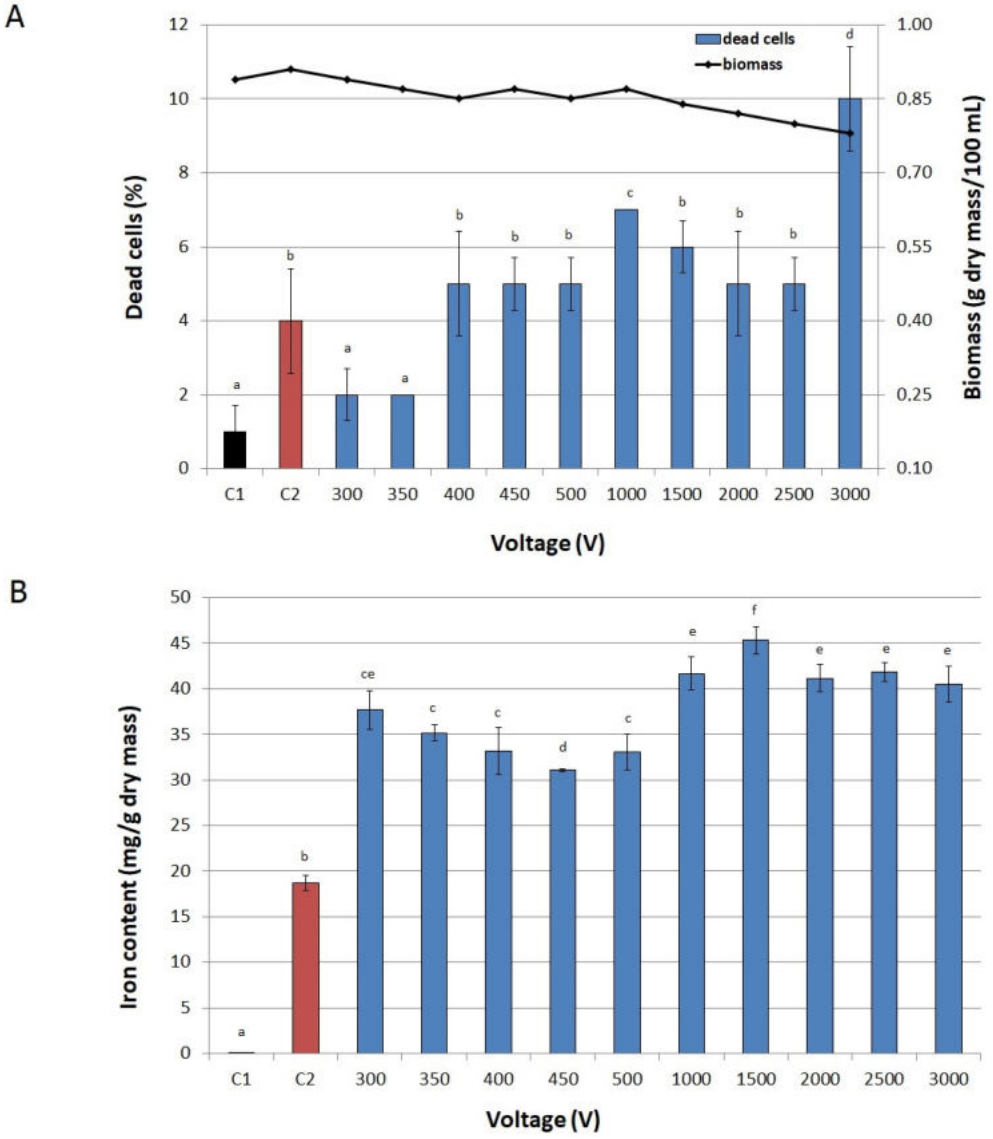

Figure 3. Effect of voltage on viability of cells and biomass (**A**) and on iron accumulation in yeast cells (**B**). C1—control culture without iron ions added to the medium and PEF treatment, C2—control culture with iron ions added to the medium (200 µg/mL) and without PEF treatment, blue bars—cultures treated with PEF (ferric nitrate, 200 µg Fe^{3+}/mL, pulse width of 10 µs, treatment time 10 min, number of pulses 600, after 20 h of cultivation). Each value is the mean ± standard deviation (n = 3). Bars with the same letter (a–f) are not significantly different ($p < 0.05$).

Application of low values of voltage (300–500 V) resulted in a twofold increase in iron content in cells compared to the control sample not exposed to PEF and with iron added to the medium (Figure 3B). The highest iron accumulation (over 2.6 times higher than in the abovementioned control sample) was achieved at 1500 V. Higher values of voltage caused a significant decrease in iron content in yeast cells, which can be related to a small decrease in biomass and yeast viability. The highest concentration of iron in cells was recorded at the pulse width of 10 µs (Figure 4B), similarly to Pankiewicz and Jamroz [23], who also observed the highest accumulation of zinc in *S. cerevisiae* cells (15 mg/g dry mass) after PEF treatment with a pulse width of 10 µs. The concentration of iron in the cells exposed to PEF at 20, 50 and 75 µs, as well as at 100, 125 and 150 µs did not differ significantly.

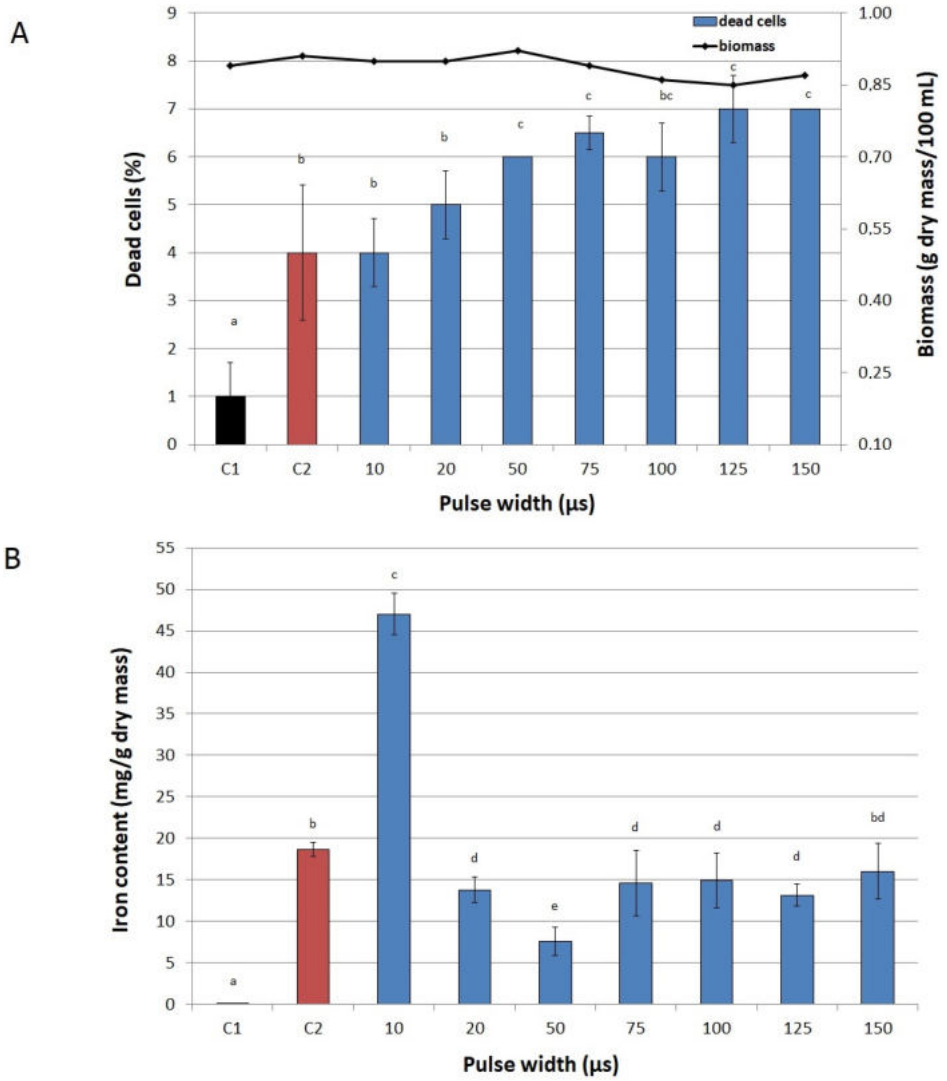

Figure 4. Effect of pulse width on viability of cells and biomass (**A**) and on iron accumulation in yeast cells (**B**). C1—control culture without iron ions added to the medium and PEF treatment, C2—control culture with iron ions added to the medium (200 µg/mL) and without PEF treatment, blue bars—cultures treated with PEF (ferric nitrate, 200 µg Fe^{3+}/mL, voltage 1500 V, treatment time 10 min, number of pulses 600, after 20 h of cultivation). The pulse width was varied from 10 µs to 150 µs at 1500 V voltage amplitude. Each value is the mean ± standard deviation (n = 3). Bars with the same letter (a–d) are not significantly different ($p < 0.05$).

Studies on the treatment time were carried out in the range of 5–20 min. Iron concentration in *S. cerevisiae* cells increased with increasing time, reaching the maximum (48.01 mg/g dry mass) at 20 min (Figure 5B). Pankiewicz and Jamroz [23] reported that the accumulation of zinc in *S. cerevisiae* cells was the highest after 15 min of PEF treatment. The difference in treatment time between our study and the studies by Pankiewicz and Jamroz [23] may be caused by the incorporation of different element into the cells of *S. cerevisiae*.

In recent years many articles on electroporation have been published using, among others, the yeast *S. cerevisiae* as a model organism. Stirke et al. [31,33] investigated the absorption of the tetraphenylphosphonium (TPP+) ion by the yeast *S. cerevisiae*. They applied electric field pulses with a duration from 5 to 150 µs and amplitude up to 10 kV/cm. The obtained results confirmed that for pulses with a duration less than 100 µs, the permeabilization is increased and no significant decrease of cell viability is observed, which is in agreement with our findings.

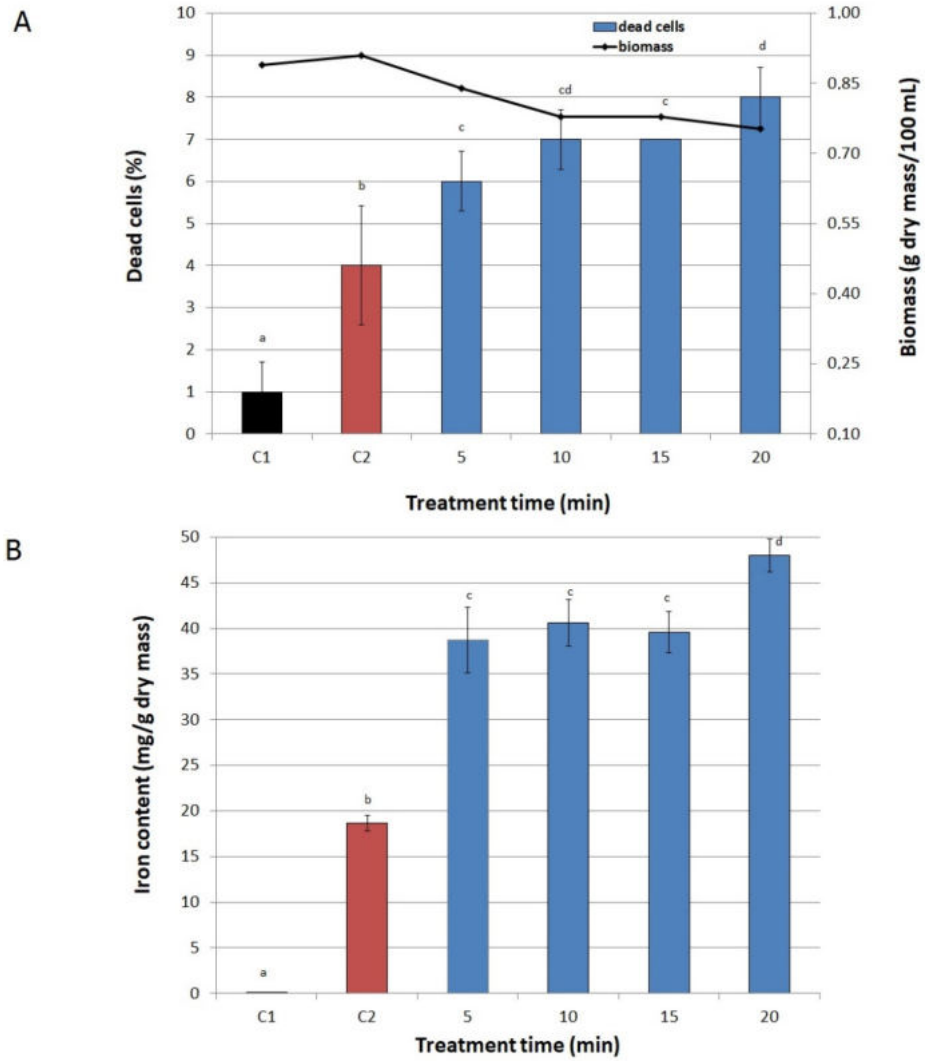

Figure 5. Effect of treatment time on viability of cells and biomass (**A**) and on iron accumulation in yeast cells (**B**). C1—control culture without iron ions added to the medium and PEF treatment, C2—control culture with iron ions added to the medium (200 μg/mL) and without PEF treatment, blue bars—cultures treated with PEF (ferric nitrate, 200 μg Fe^{3+}/mL, voltage 1500 V, pulse width of 10 μs, after 20 h of cultivation). Each value is the mean ± standard deviation (n = 3). Bars with the same letter (a–d) are not significantly different ($p < 0.05$).

Also the assumption that the influence of the PEF on the yeast cell wall increases its permeability to TPP+ was confirmed. This process can be controlled by appropriately setting the amplitude and duration of the PEF. The authors also concluded that the similar characteristic lifetimes of the non-equilibrium pores in the cell wall and membrane after PEF treatment indicate a strong coupling between these parts of the cell. Experiments carried out on Chinese hamster lung fibroblast cells (DC-3F) and human adipose mesenchymal stem cells (haMSC) in the medium containing Ca^{2+} using microsecond pulsed electric fields confirmed the influx of extracellular calcium induced by the electric pulse as a result of the electropermeabilization of the cell membrane [34]. The final step in our experiment was setting the cultivation time after which yeast cells were exposed to PEF. We considered the optimal time after which the highest biomass production was achieved. In our case it was 20 h (0.92 g dry mass/100 mL) (Figure 6), similarly to Pankiewicz et al. [12] and Pankiewicz and Jamroz [14], who treated *S. cerevisiae* cultures with PEF to improve, respectively, the simultaneous accumulation of magnesium and zinc, and accumulation of selenium in biomass. However, there are considerable discrepancies in the optimal time of cultivation set in studies on the accumulation of iron from the medium by yeasts, which may result

from different culture conditions. For instance, Stehlik-Thomas et al. [35] reported that the highest concentration of iron in cells (10 mg/g dry yeast biomass) was obtained after 12 h of cultivation in anaerobic conditions. On the other hand, under semiaerobic conditions, the highest accumulation was achieved after 16 h of cultivation, but it was four times lower (2.5 mg/g dry yeast biomass) than the above-mentioned one. Wang et al. [36], in turn, obtained the highest Fe content (7.854 mg/g dry mass) in yeast cultivated for 60 h at 30 °C.

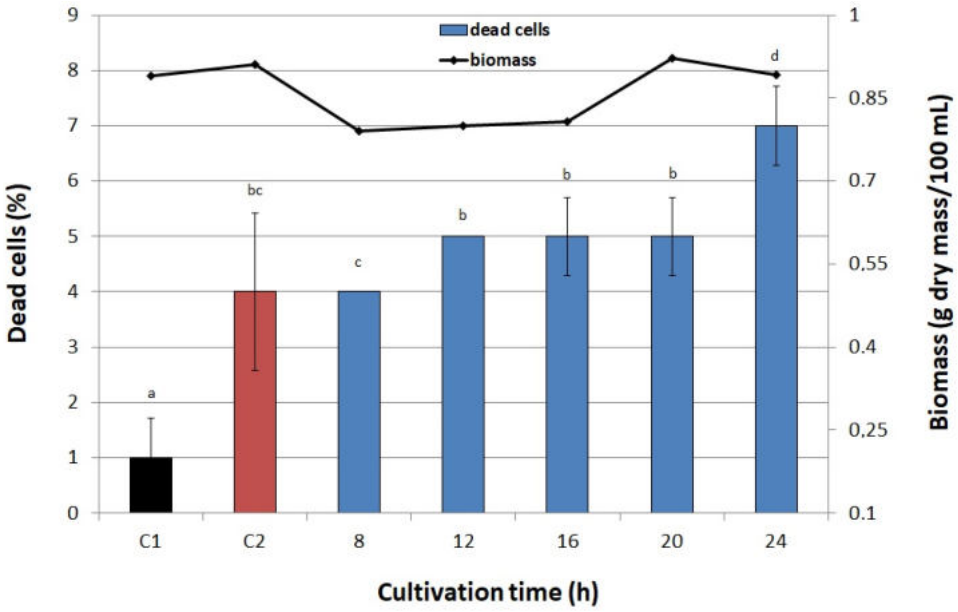

Figure 6. Effect of cultivation time after which PEF was applied on viability of cells and biomass. C1—control culture without iron ions added to the medium and PEF treatment, C2—control culture with iron ions added to the medium (200 μg/mL) and without PEF treatment, blue bars—cultures treated with PEF (ferric nitrate, 200 μg Fe^{3+}/mL, voltage 1500 V, pulse width 10 μs, treatment time 20 min, 1200 pulses). Each value is the mean ± standard deviation (n = 3). Bars with the same letter (a–d) are not significantly different ($p < 0.05$).

2.3. Fluorescence Imaging of Yeast Cells

Figure 7 presents the images of yeast cells stained with Rhodamine B and observed under a fluorescence microscope. This dye crosses cell membranes and is captured by mitochondria without inducing cell lysis. Rhodamine-based dyes can be used to bioimage iron pools in living cells [37]. Almost all cells from the control sample not subjected to PEF and without iron supplementation were dark (Figure 7A), whereas those from the sample enriched with iron without PEF partly showed green fluorescence (Figure 7B). PEF treatment enhanced the accumulation of iron in yeast cells, so almost the entire population of cells showed strong green fluorescence (Figure 7C). The detected fluorescence emission is caused by the iron complexation-induced opening of the spirocyclic ring of the rhodamine-based probes [37] and its relative intensity is proportional to the iron concentration [38]. In our previous studies [39] we have observed yeast cells enriched with calcium and zinc by PEF treatment and stained with, respectively, calcium orange and morin under a confocal laser microscope. The experiments were carried out with the same PEF treatment system as this used here. We have done semi-quantitative analysis of ions within the limits of the cells. The study revealed that that fluorescence inside cells from control samples (without ions added to the medium and PEF treatment) was lower than that observed for cells from the sample enriched with calcium and zinc using PEF. On the basis of the optical sections, we also have made the 3D reconstructions of ion-rich areas distribution in the cell. It has been confirmed that in PEF-treated yeast cells absorption of calcium and zinc ions was higher and that metal ions were distributed inside the cell.

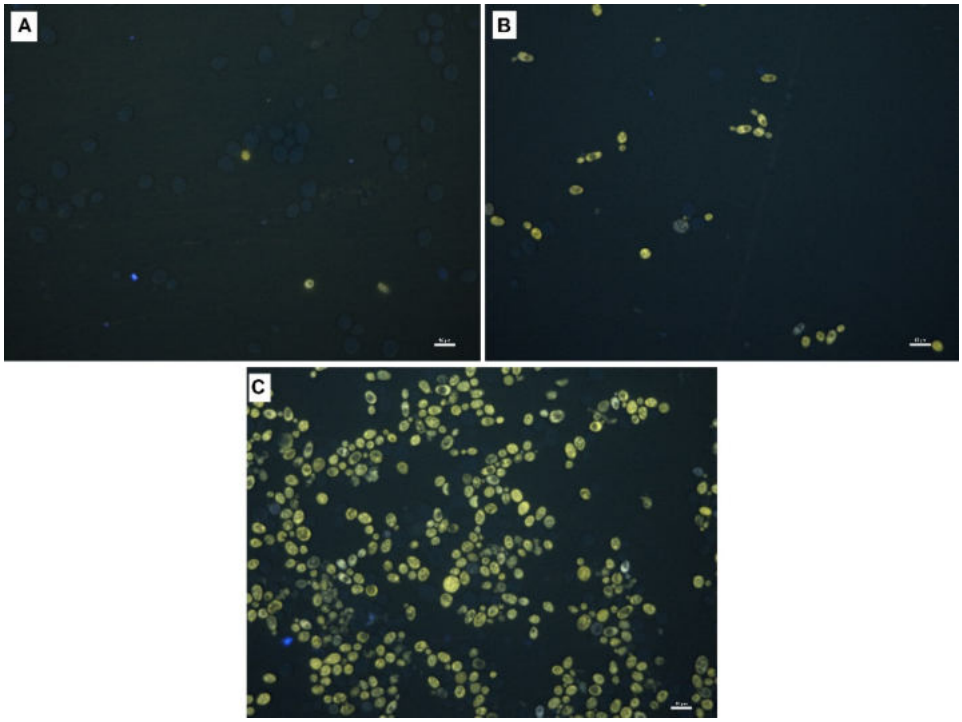

Figure 7. Fluorescent microscopy images of yeasts stained with Rhodamine B: (**A**) C1—control culture without iron ions added to the medium and PEF treatment, (**B**) C2—control culture with iron ions added to the medium (200 μg/mL) and without PEF treatment, (**C**) culture with iron added to the medium (200 μg/mL) and PEF treatment (ferric nitrate, voltage 1500 V, pulse width 10 μs, treatment time 20 min, 1200 pulses, after 20 h of cultivation). The scale bars correspond to 10 μm.

2.4. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy

FTIR spectroscopy is a non-destructive and an excellent tool for rapid studying of a fingerprint of the biological samples under investigation, giving information on its most important biochemical components such as proteins, lipids, nucleic acids, and carbohydrates, cell wall and membranes. The ATR-FTIR spectra of lyophilized yeast cells stained from the control sample (C1), the iron-enriched samples both without PEF treatment (C2) and with the use of PEF at optimal conditions (Fe + PEF) were recorded in the region between 4000 and 750 cm^{-1}. The location of the characteristic bands corresponds to the data reported in the literature [40–44], (Figure S1, Table S1).

The broad peak at about 3290 cm^{-1} indicated the presence of both amine (N–H) and bonded hydroxyl (O–H) groups (Figure S1). The lipid region between 2800 and 3000 cm^{-1} is dominated by the C–H symmetric and asymmetric stretching vibrations of the CH_2 and CH_3 groups assigned to fatty acid chains in phospholipid membranes and the cell wall (Figure 8A). The spectra between 1700 and 1500 cm^{-1} are dominated by the amide I and amide II bands, respectively due to the C=O stretching and the N–H bending of the peptide bond (Figure 8C). The amide I band particularly provides information on the protein secondary structure due to the decomposition in several subbands characteristic of the different protein conformations: α-helix (~1656 cm^{-1}), and β-sheet (1627–1635 cm^{-1}) [45]. The bands within the region of 1200–900 cm^{-1} are associated with the mixed vibrations of polysaccharides (C–O–H and C–O–C of the mannan and glucan vibrations), nucleic acids (P=O), and lipids (C-H bending modes) [41,46] (Figure 8E). To better estimate possible spectral changes due to iron accumulation in yeast cells, the second order derivative spectra have been generated to resolve the overlapping bands into individual ones, thus increasing the accuracy [47]. The predominant bands acquired in second derivative spectra of examined samples and their attribution to specific chemical groups are summarized in Table 1. The second derivative spectra of yeast cells from the C1, C2 and Fe + PEF samples

were determined in the three significant ranges, which are dominated by bands associated with the absorption modes of lipids, proteins and carbohydrates (Figure 8B,D,F).

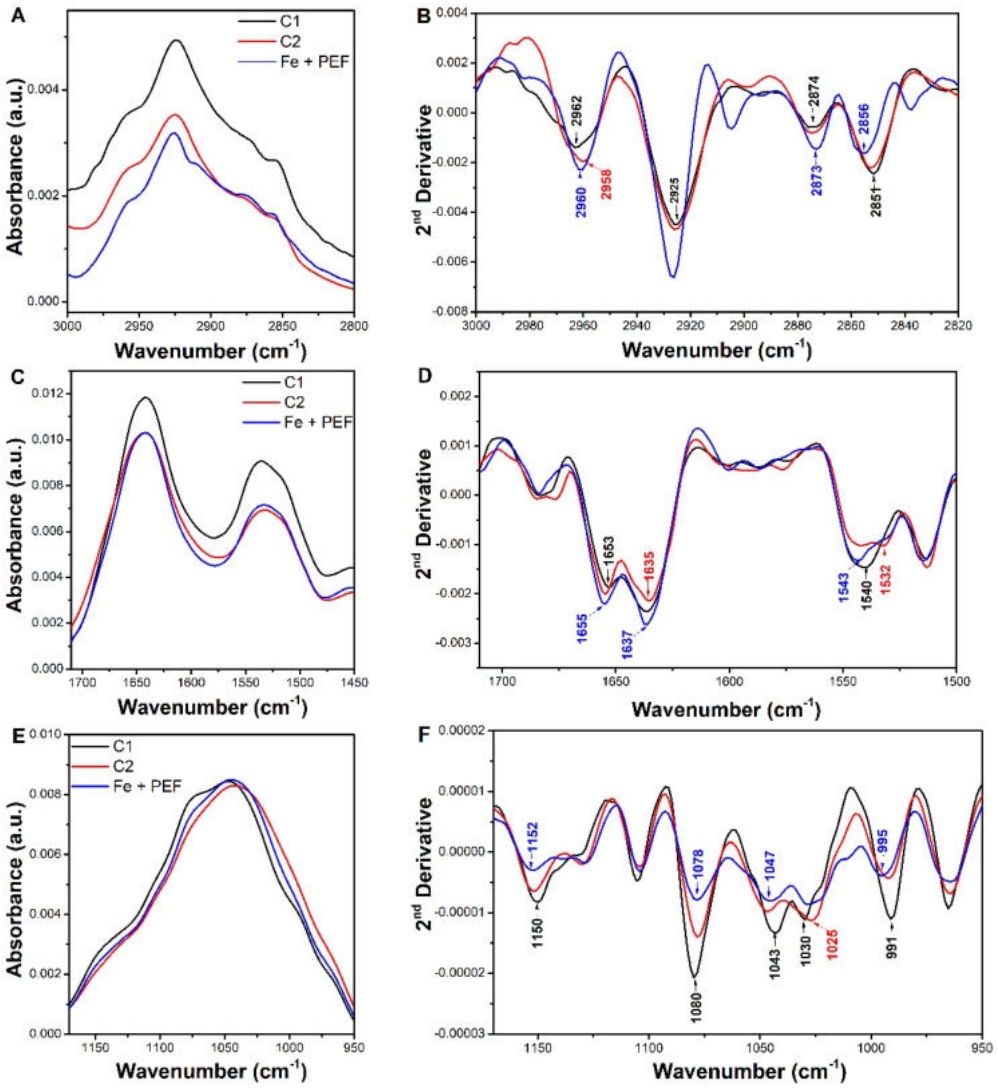

Figure 8. The ATR FTIR spectra of yeasts stained and their second derivatives in the selected ranges. (**A**,**B**): the region of the vibrational modes mainly due to the asymmetric and symmetric stretching vibrations of CH_2 and CH_3 methylene groups (**C**,**D**): the region of amide I and amide II bands; (**E**,**F**): the region of carbohydrates modes. The second derivative spectra have been normalized to the CH_2 band at ~2924 cm^{-1} (**B**), to the amide I (**D**) while in panel F spectra have been normalized at the area under range 1180-900 cm^{-1}. C1-control culture without iron added to the medium and PEF treatment (black line), C2-control culture with iron ions added to the medium (200 μg/mL) and without PEF treatment (red line), and Fe + PEF culture with iron ions added to the medium (200 μg/mL) and treated with PEF (ferric nitrate, voltage 1500 V, pulse width 10 μs, treatment time 20 min, 1200 pulses, after 20 h of cultivation) (blue line).

As can be noted, the changes in the spectral features of the lipid region (3000–2800 cm^{-1}) are related to a substantial increase in intensity of the CH_2 bands at 2925 cm^{-1} in case of the samples subjected to PEF (Figure 8B). Moreover, a weaker increase in the intensity of the CH_3 absorption at ~2960 and ~2873 cm^{-1} has been also detected for both the culture iron-supplemented samples. The variation of the CH_2 and CH_3 contribution could be related to the inducing change in yeast membrane fluidity by the means of iron ions modifications. In addition, a 1–4 cm^{-1} upward shift of the CH_2 asymmetric and symmetric stretching bands was observed especially in yeast subjected to PEF (Figure 8B). Indeed, the state of lipid cell membranes are related to the spectral shift of the CH_2 bands into lower or higher frequencies and correspond, respectively, to their rigidity or fluidity [47] Ganeva

et al. [48] and Strike et al. [31] have postulated that yeast treatment with PEF could induce not only the membrane permeabilization, but also cause the changes in the structure of cell wall, leading to enhance the yeast cell wall porosity.

Table 1. The most important second derivative bands obtained in the spectra of all yeast samples, and the type of vibrations with assigned cellular components.

Wavenumber (cm^{-1})			Assignment of the Type of Vibrations
C1	**C2**	**Fe + PEF**	
2962	2958	2960	$\nu_{as}(CH_3)$ of lipids
2925	2925	2926	$\nu_{as}(CH_2)$ of lipids
2851	2856 *	2856 *	$\nu_s(CH_2)$ of lipids
1653	1654	1655	80% ν(C=O), 20% ν(C–N) of amide I, τ(HOH) of water
1635	1635	1637	
1540	1543 *	1543 *	60% τ(N–H), 30% ν(C–N), 10% ν(C–C) of amide II
1152	1152	1150	ν(C–O) of carbohydrates (mannans and β-1,3 glucans)
1080	1078	1078	$\nu_{as}(PO_2^-)$ of membrane lipids, carbohydrates
1043	1047 *	1047 *	$\nu_s(PO_2^-)$, mannans
1030	1025 *	1025 *	β-1,4 glucans
991	993 *	995 *	β-1,6 glucans

The symbols concerning the vibrations assignment are related to the stretching vibrational mode (ν), deformational (δ); bending (τ), and symmetrical (s) and asymmetrical (as) modes. An assignment of spectral features was collected according to the literature [41,42]. Asterisks (*) indicate the most significant spectral shifts (>2 cm^{-1}) between the control sample (C1) and the iron-enriched samples both without PEF treatment (C2) and with the use of PEF (Fe + PEF).

When the yeast cells from the control culture and its metal modified species are compared, the second derivative profiles of amide I and II are quite similar except for the position of the bands (Figure 8D). Indeed, the peak position at 1653 cm^{-1} (assigned to α-helix) and 1635 cm^{-1} (assigned to β-sheets and most likely intracellular water) are affected both in the case of C2 and Fe + PEF which was seen in marginal frequency shifts, and variations in absorbance intensities, compared with yeast from the control culture C1. This shifting of the amide I band to 1655 cm^{-1} was related to the involvement of the O and N atoms of the polypeptide chain in iron ions' binding (Figure 8D).

The changes of a broad peak shape in the region 1180–950 cm^{-1} for metal-modified yeast in comparison to the control yeast sample may indicate the interactions of iron ions with polysaccharides present in the yeast cell wall. Indeed, the majority of polysaccharides of the yeast cell is found in its wall [49]. More specifically, the inner wall layer consists of mainly β-1,3-glucan but the outer one is formed by highly glycosylated mannoproteins with numerous phosphate groups in their carbohydrate side chains, resulting in a net negative surface charge. In fact, in the second derivative spectra of yeast after PEF treatment, a band attributed to β-1,3-glucan (1150 cm^{-1}) is much less intense, broadened and shifted to a higher frequency suggesting its participation in iron ions' binding. Taking into account other carbohydrate-related bands, a decrease in intensity of the bands assigned to the glucan structure, namely, at 1080 cm^{-1}, 1043 cm^{-1} (mannans), 1030 and 991 cm^{-1} (β-1,6 glucans), and accompanied with their spectral shift compared to control C1 was also observed (Figure 8F). Finally, a strong reduction of the band at about 1080 cm^{-1} observed in the yeast sample after PEF treatment may suggest the interaction of the PO_2^- of membrane phospholipids with the positive iron ions. The reduction of the abovementioned band intensities can be explained by iron ions binding to yeast cells as a result of presence of bond stretching to a lesser degree [50], so the iron ions could pass through the cell wall and periplasmic space and reach the surface of the plasma membrane to a large extend by PEF treatment.

2.5. Fermentative Properties and Protein Content in Yeast Cells

In this study we also investigated the effect of PEF on the fermentation properties of yeast. Figure 9 shows the results obtained for the controls C1 and C2, and for the sample

obtained at the optimal PEF conditions (Fe + PEF). The control C1 showed the highest fermentative activity. Already in the 30th minute of the test, dough growth was observed, while the dough with the addition of yeast with C2 and Fe + PEF started to rise only after 60 min. After 120 min the volume of dough containing yeast from the control C1 was 1.7-fold higher than that with yeast from the control C2 and 1.85-fold higher than in the case of dough with yeast exposed to PEF. The decrease in fermentative activity of the control C2 and the PEF-exposed sample (Fe + PEF) compared to C1 can be partly explained by their lower content of protein (Table 2). Another factor influencing yeast fermentation is the availability of assimilable nitrogen. Generally, *S. cerevisiae* yeasts are unable to use nitrate as sole nitrogen source [51] because, being deprived of molybdenum-dependent enzymes, they cannot assimilate it [52].

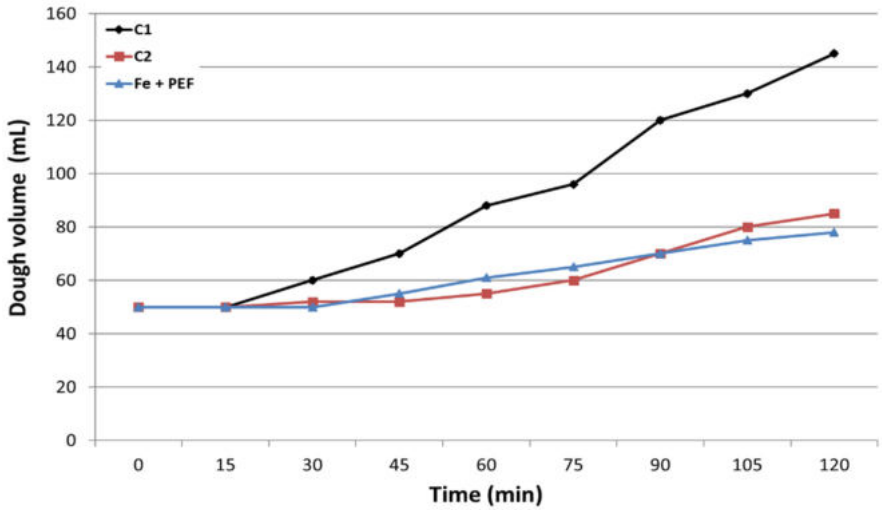

Figure 9. Effect of PEF and iron enrichment on fermentative properties of yeast: C1—control culture without iron added to the medium and PEF treatment, C2—control culture with iron ions added to the medium (200 μg/mL) and without PEF treatment, Fe + PEF—culture with iron ions added to the medium (200 μg/mL) and treated with PEF (ferric nitrate, voltage 1500 V, pulse width 10 μs, treatment time 20 min, 1200 pulses, after 20 h of cultivation). Each value is the mean ± standard deviation (n = 3).

Table 2. Protein content in yeast: C1—without the addition of iron ions and not subjected to PEF; C2—with the addition of iron ions (200 μg/mL) and not subjected to PEF, Fe + PEF—with the addition of iron ions (200 μg/mL) and subjected to PEF (ferric nitrate, voltage 1500 V, pulse width 10 μs, treatment time 20 min, 1200 pulses, after 20 h of cultivation). Each value is the mean ± standard deviation (n = 3). Means with the same letter (a–c) are not significantly different ($p < 0.05$).

Sample	Protein Content (%)	Iron Content (mg/g Dry Mass)
C1	59.13 ± 0.18 ^{a}	0.13 ± 0.01 ^{a}
C2	58.24 ± 0.35 ^{b}	18.68 ± 0.86 ^{b}
Fe + PEF	54.07 ± 0.11 ^{c}	48.01 ± 0.88 ^{c}

The study of Święciło [53] also demonstrated that *S. cerevisiae* show low sensitivity to sodium nitrate (V) which was explained by the fact that nitrates (V) are removed from cells efficiently. The author observed a 50% death rate of the yeast population when the concentration of this salt was as high as about 1 mol/L. The lower fermentation performance of yeasts from the sample C2 and that treated with PEF may also be related to the presence of high concentration of iron in cells. Iron, along with potassium, magnesium, calcium, manganese, copper and zinc, is one of the most important metals that influence yeast fermentation processes [17]. Excess iron can be detrimental to cells as certain forms can be involved in Fenton redox reactions that accelerate the formation of reactive oxygen

species (ROS), such as hydroxyl radicals that damage cells at the level of membranes, proteins and nucleic acids [54]. Oxidative damage to proteins is expected to have negative consequences on the fermentative ability of yeast [55].

3. Materials and Methods

3.1. Microorganism and Growth Media

The industrial strain of *S. cerevisiae* 11 B1 from the Yeast Plant (Lublin, Poland) was used. The composition of medium for agar slants and inoculum growth, as well as that used in the experiment with PEF is presented in Table 3. The pH was adjusted to 5. All reagents were of analytical grade purity.

Table 3. The composition of media used in the experiment.

Medium for Inoculum Growth	Concentration (g/L)
Sucrose (POCH, Gliwice, Poland)	20
NH_4Cl (POCH, Gliwice, Poland)	3.2
KH_2PO_4 (POCH, Gliwice, Poland)	2.5
Na_2SO_4 (POCH, Gliwice, Poland)	2.0
$MgCl_2 \bullet 6H_2O$ (POCH, Gliwice, Poland)	1.5
Yeast extract (BTL, Łódź, Poland)	5.0
Agar (DIFCO, Detroit, MI, USA)	15.0
Unhopped wort (Lublin Breweries S.A., Lublin, Poland)	40 mL
Experimental medium	
Peptone (Sigma–Aldrich CO, St. Louis, MO, USA)	10.0
Yeast extract (BTL, Łódź, Poland)	5.0
Glucose (POCH, Gliwice, Poland)	10.0

3.2. Biomass Cultivation

The yeast was passaged three times on agar slants, grown for 48 h in a thermostat at 30 °C, and finally used for the inoculum preparation. Cells from one slant were used to inoculate 150 mL of sterile medium in an Erlenmeyer flask. Cultures were grown in a shaking incubator (NBB 205L, N-BIOTEK Inc., Gyeonggi-Do, Korea) at 30 °C and 100 rpm for 48 h.

The culture medium was centrifuged after 48 h of culture and the cell pellet was washed three times with sterile water. The pellets from three Erlenmeyer flasks were collected and resuspended in sterile water to a final volume of 300 mL. 10 mL of the thus prepared inoculum was used to inoculate the immersion cultures into 500 mL Erlenmeyer flasks, each containing 90 mL of medium. Growth conditions were identical to the inoculum.

3.3. Design of Experiments for Optimization Iron Accumulation

Process parameters were optimized by maintaining all factors at a constant level except the one under study (one-factor-at-a-time (OFAT) method). The selection of parameter values was based on the previous studies by Pankiewicz and Jamroz [11]. The following parameters were optimized: iron concentration, voltage, pulse width, treatment time and cultivation time. Iron salt was selected in a separate experiment.

3.4. PEF Treatment

The cultures of *S. cerevisiae* were grown in flasks under continuous agitation for 20 h and then treated with PEF using an ECM 830 unipolar square wave generator (BTX Harvard Apparatus, Holliston, MA, USA). A culture with a volume of 100 mL was placed in the PEF treatment chamber consisted in a beaker (300 mL) and four parallel stainless steel electrodes of an area equal to 4 cm^2, opposed to each other with a spacing of 5.1 mm (Figure 10), mounted on a removable cover. The conductivity of the culture medium was 2.6 mS/cm (conductometer CC-505, Elmetron, Zabrze, Poland) and the frequency for delivering pulses was 1 Hz. While delivering the pulses the solution was being mixed with

the help of a rotating magnet (100 rpm) to avoid cells sedimentation. The electrodes were immersed for approximately 7.4 mm into the solution. The temperature was monitored during PEF treatment.

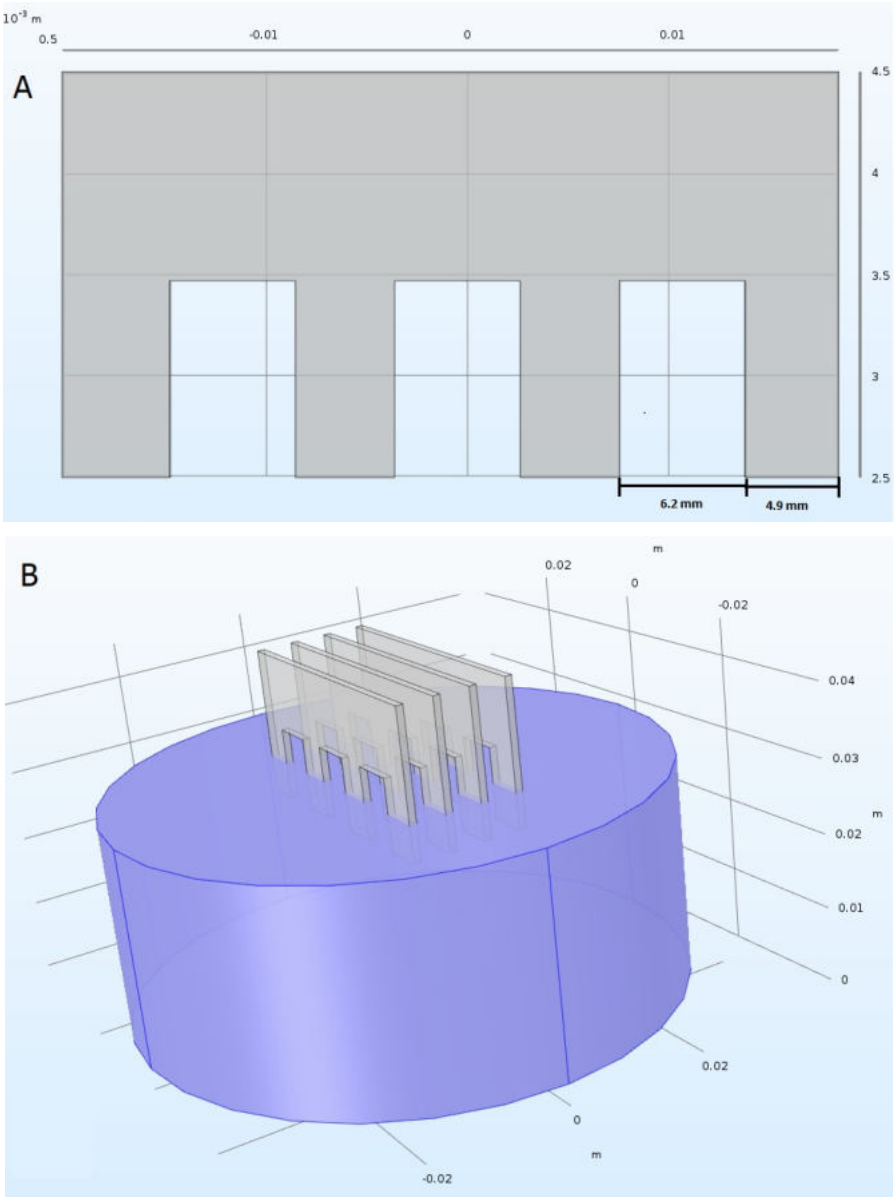

Figure 10. Schematic representation of a single electrode (**A**) and set of electrodes immersed into the culture medium (**B**).

3.5. Selection of Iron Salt

In order to select the iron salt with the highest accumulation in yeast cells five iron salts ($FeCl_2 \bullet 4H_2O$, $Fe_2SO_4 \bullet 6H_2O$, $FeCl_3 \bullet 6H_2O$, $Fe(NO_3)_3 \bullet 9H_2O$, and iron (III) citrate) were added to the medium in concentration of 100 µg Fe/mL. The conductivity of the solution was 2.7 mS/cm. Cultures after 20 h cultivation were exposed to PEF with the following initial conditions: voltage of 1500 V, pulse width of 10 µs, treatment time 10 min, number of pulses 600. Then cultures were centrifuged, washed several times with deionized water, and lyophilized in a Model 64132 freeze dryer (Labconco, Kansas City, MO, USA). At the same time, a control sample (C1) without iron in the medium and PEF treatment was prepared.

3.6. Selecting Optimal Process Parameters

3.6.1. Iron Concentration in the Medium

The freshly prepared solution of selected iron salt was added to the medium just before electroporation so that the concentration of iron ions in a sample was, respectively, 100, 200, 300, and 400 μg/mL and conductivity of the solutions was in the range 2.7–3.0 mS/cm, respectively. Then the culture was subjected to PEF with the same parameters as described in Section 3.4. Simultaneously a control sample C1 was prepared.

3.6.2. Voltage

After selecting the iron concentration, voltage was screened in the range 300–3000 V at the constant pulse width of 10 μs, treatment time of 10 min, and number of pulses 600. Two untreated samples—one with no iron ions in the medium (C1) and the other with 200 μg Fe^{3+}/mL (C2), served as controls.

3.6.3. Pulse Width

Pulse width was tested for the values of 10, 20, 50, 75, 100, 125 and 150 μs at the constant voltage of 1500 V, treatment time of 10 min, and number of pulses 600. Two untreated samples—one with no iron ions in the medium (C1) and the other with 200 μg Fe^{3+}/mL (C2), served as controls.

3.6.4. Treatment Time

In the final step, treatment time was varied for 5, 10, 15, and 20 min with constant values of other parameters. During the treatment time pulses were delivered at 1 Hz pulse repetition frequency. Two untreated samples—one with no iron ions in the medium (C1) and the other with 200 μg Fe^{3+}/mL (C2), served as controls.

3.6.5. Cultivation Time

Cultures were subjected to PEF after 8, 12, 16, 20, and 24 h of cultivation. The following PEF parameters were applied: voltage of 1500 V, pulse width 10 μs, treatment time 20 min, 1200 pulses. Two untreated samples—one with no iron ions in the medium (C1) and the other with 200 μg Fe^{3+}/mL (C2), served as controls.

After each stage of experiment cells were centrifuged, washed several times with deionized water, and then lyophilized in a Model 64132 Labconco freeze dryer. The experiment was performed in triplicate.

3.7. Determination of Iron Concentration

Iron concentration was determined using the flame atomic absorption spectrophotometry (FAAS, Solaar 939, Unicam, Cambridge, UK). Samples of the freeze-dried biomass were weighed into the thimbles, flooded with 3 mL of HNO_3–$HClO_4$ (5:1) mixture and mineralized for 20 min at 250 °C in a microwave oven (MARS 5, CEM Corporation, Matthews, NC, USA). After cooling, solutions were transferred to 10 mL measuring flasks and topped up with deionized water [11]. The determination was performed in triplicate.

3.8. Determination of Yeast Biomass and Cell Viability

Cell biomass was estimated by measurement of optical density at 600 nm against pure culture medium in 2-mm measurement cells. Then dry mass was calculated using equation for the standard curve. In the case of cell viability, dead cells were counted in the Thoma chamber after staining with the 0.01% methylene blue solution. Both determinations were performed in triplicate.

3.9. Determination of Fermentative Properties and Protein Content in Yeast Cells

Fermentative activity of yeast was determined by measuring the volume of the expanding dough over time (0–120 min) [56]. Protein content in yeast cells was determined by the Kjeldahl method [57]. The results are means of three measurements.

3.10. Fluorescent Microscopy

Yeast cells from the samples C1 and C2, and the sample enriched with iron at the optimal PEF conditions were stained with an 0.01% ethanol solution of Rhodamine B (Merck, KGaA, Darmstadt, Germany) and observed under a fluorescent microscope (Eclipse 90i, Nikon, Tokyo, Japan). The excitation and emission wavelengths were 550 nm and 580 nm, respectively.

3.11. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy

Mid-infrared absorption spectra were acquired by Attenuated Total Reflectance-Fourier Transform Infrared spectroscopy (ATR-FTIR) IRSpirit (Shimadzu, Kyoto, Japan) equipped with a DLATGS detector. The measurements were performed in attenuated total reflectance mode using the QATR™-S Single-Reflection ATR Accessory with a Diamond Crystal (Shimadzu). A pinch of dried samples were placed directly onto the crystal (with a contact area diameter of 1.8 mm) and pressured against its surface with a swing clamp mechanism. Spectra were collected with 36 spectral scans at a resolution of 4 cm^{-1} within the wavenumber range between 4000 and 500 cm^{-1}. The spectra were ATR, air vapour and baseline corrected and normalized. The spectral normalization was performed in terms of the equal area in the appropriate spectral range (3000–2800 cm^{-1} for lipids, 1720–1500 cm^{-1} for proteins, 1180–900 cm^{-1} for carbohydrates). To gain more insight into cell structural components, second derivation procedure was performed. All spectral and data analysis were performed using the Grams/AI 8.0 software (Thermo Scientific, Waltham, MA, USA).

3.12. Data Analysis

Regression analysis and significance tests were performed using the Statistica 13.3. software (StatSoft, Inc., Tulsa, OK, USA). The pos-hoc Tuckey test was employed to determine differences between means. Results of $p < 0.05$ were considered statistically significant.

4. Conclusions

This study showed that pulsed electric field increases iron accumulation in *S. cerevisiae* cells. At the iron ion concentration of 200 μg/mL and under the following PEF conditions: voltage of 1500 V, pulse width of 10 μs, treatment time 20 min, number of pulses 1200, as well as the optimal cultivation time of 20 h, the amount of accumulated iron increased from 18.68 mg/g dry mass (for the control culture supplemented with iron but not treated with PEF) to 48.01 mg/g dry mass. At the same time, treatment with PEF did not significantly influence biomass production and cell viability. FTIR analysis of unloaded and iron ions loaded yeast cells in the range of 4000–900 cm^{-1} allowed us to determine the presence of functional groups that could be involved in iron ion binding. Yeast treatment with PEF simultaneously induces the permeabilization of the whole cell barrier, both the cell wall and the membrane. This is responsible for the increase of the iron ions adsorption on the surfaces of negatively charged yeast cells and the interaction between it and the positive iron ions. The elevated amount of iron in cells caused, however, over 40% decrease in the fermentative activity of yeast as compared to the control sample. Overall iron-enriched yeast may be considered an additional source of this element in a diet.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biom11060850/s1, Figure S1: ATR FTIR spectra of all yeast samples (*S. cerevisiae*) investigated acquired in the wavenumber range from 4000 to 750 cm^{-1}.The most prominent absorbance bands are indicated and correspond to the stretching vibrations of CH_2 and CH_3 groups in lipids, amide I (~1640 cm^{-1}) and amide II (~1530 cm^{-1}) bands of proteins and to the carbohydrate region between 1200 and 900 cm^{-1}. Each spectrum presented is an average of three independent measurements and normalized for equal area between 1180 and 900 cm^{-1}, Table S1: The most important bands obtained in the spectra of yeast, and the type of vibrations with assigned sample components.

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The Use of Iron-Enriched Yeast for the Production of Flatbread

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Abstract: The most common cause of iron deficiency is an improperly balanced diet, in which the body's need for iron cannot be met by absorption of this element from food. Targeted iron supplementation and food fortification may be the main treatments for iron deficiency in the population. However, many iron-rich supplements and foods have low bioavailability of this element. In our study, we used yeast enriched with iron ions to produce flatbread. The yeast cells accumulated iron ions from the medium supplemented with $Fe(NO_3)_3 \cdot 9H_2O$, additionally one of the cultures was treated with pulsed electric field in order to increase the accumulation. The potential bioavailability of iron from flatbread containing 385.8 ± 4.12 mg of iron in 100 g dry mass was 10.83 ± 0.94%. All the flatbreads had a moderate glycemic index. There were no significant differences in antioxidant activity against DPPH$^{\bullet}$ between flatbread with iron-enriched and non-iron-enriched yeast. Sensory evaluation showed that this product is acceptable to consumers since no metallic aftertaste was detected. Iron enriched flatbread can potentially be an alternative to dietary supplements in iron deficiency states.

Keywords: iron deficiency; PEF; flatbread

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1. Introduction

Iron deficiency is a global health problem that affects people of all ages. This condition can also accompany many diseases. The etiology of iron deficiency is variable and depends on many factors that reduce iron absorption and increase the demand for this element [1]. Increased demand is a physiological condition and is commonly observed in infants, preschool children, spikes in adolescents during adolescence, and in pregnant women (mainly in the second and third trimesters) [2]. Reduced iron intake may be a direct consequence of malnutrition such as that of children and pregnant women in poor countries, or it may be attributed to a vegan or vegetarian diet that is low in iron [3]. Reduced iron absorption occurs in the presence of inhibitors such as calcium, phytates (present in grains), and tannins (present in tea and coffee). In addition, this problem also occurs after surgery that increases the pH of the stomach, which reduces conversion to ferrous ions. Diseases such as *Helicobacter pylori* infection, celiac disease and intestinal inflammation also cause reduced iron absorption [4].

Iron performs many important functions in the human body. Its primary role is to participate in the transport of oxygen through erythropoiesis, therefore, in patients with chronic inflammation, iron deficiency may be particularly severe and may exacerbate the disease state [5]. Additionally, iron deficiency is a common cause of anemia since iron is an integral part of the blood protein-hemoglobin (Hb) [6].

One way to treat iron deficiency is through oral supplementation. However, the use of dietary supplements is associated with the risk of side effects, the most common of which are gastrointestinal symptoms: Nausea, vomiting, abdominal pain, constipation, flatulence, diarrhea, occurring in up to 40% of patients [7]. Additionally, these preparations are characterized by low absorption of iron in the intestines and can have a metallic taste. For these reasons, there is a need for an effective, long-term strategic approach. In this

context, food fortification with iron remains a promising and cost-effective approach to treating iron deficiency [6].

Prevention or treatment of iron deficiency can be achieved by enriching microorganisms (for example, yeast) used in the food industry with iron ions through the application of pulsed electric field (PEF) [8]. Previous studies have shown that using this technique increases the efficiency of ion accumulation by yeast from the medium [9–14]. Higher ion accumulation in cells results from the increased permeability of the cell membrane due to the phenomenon of electroporation. Electroporation consists of the development of structural defects in lipid bilayer membranes caused by the externally applied PEF [15]. It has been hypothesized that the defects are in the form of metastable nanoscale pores through which small molecules and ions could pass [15]. Metal ions adsorbed on the cell's surface may next be a subject of intracellular bioaccumulation. This way yeasts produce metal-protein complexes called metalloproteins (or bioplexes), which are highly absorbed by the human body [16,17].The yeast biomass enriched with iron using PEF could be used for the production of functional food.

Therefore, in our research, we produced flatbread with the addition of yeast enriched with iron ions using two methods: Only by adding iron salt to the nutrient medium and additionally supporting the accumulation by the action of pulsed electric field. Our main goal was to investigate the potential bioavailability of iron from such prepared flatbread and to examine the nutritional and antioxidant properties of this food product.

2. Results and Discussion

2.1. Nutrient Composition and Glycemic Index of Flatbreads

Table 1 presents the nutritional value and glycemic index of flatbreads produced with the addition of yeast from the cultures not supplemented and supplemented with iron by two different methods. The nutritional value of the flatbread depends mainly on the chemical composition of the flour and other ingredients used in its preparation [18]. Carbohydrates were in the range 58–62% and constituted the highest content in all analyzed samples. Samples did not differ significantly in terms of protein and fat contents.

Table 1. Nutritional composition, caloric value, and glycemic index (GI) of flatbreads(content given on a dry mass) produced with yeast not enriched and enriched with iron ions.

Flatbread with Yeast	Proteins (%)	Fat (%)	Carbohydrates (%)	Ash (%)	Caloric Value (kcal/100g)	Caloric Value (kJ/100g)	Glycemic Index (IG)
C1	11.85 ± 0.42 ^{a}	4.50 ± 0.33 ^{a}	62.09 ± 0.48 ^{c}	21.56 ± 0.19 ^{a}	336.23 ± 2.38 ^{c}	1406.79 ± 9.97 ^{c}	56.24 ± 0.12 ^{a}
C2	11.44 ± 0.14 ^{a}	4.81 ± 0.18 ^{a}	59.42 ± 0.18 ^{b}	24.33 ± 0.33 ^{b}	326.70 ± 2.18 ^{b}	1366.91 ± 9.11 ^{b}	56.51 ± 0.25 ^{a}
P	12.39 ± 0.45 ^{a}	4.11 ± 0.19 ^{a}	58.14 ± 0.40 ^{a}	25.37 ± 0.35 ^{c}	319.07 ± 2.26 ^{a}	1334.97 ± 9.45 ^{a}	56.23 ± 1.34 ^{a}

C1—yeast without the addition of iron ions and without PEF; C2—yeast with the addition of iron ions and without PEF; P—yeast with the addition of iron ions and PEF. Each value is the mean ± standard deviation (n = 3). Results with the same letter within a column are not significantly different ($p < 0.05$).

The flatbread samples differed significantly in the ash content, which was caused by the higher content of iron in the samples enriched with this element. The amount of carbohydrates, fats, and proteins influenced the caloric value of the products. There were statistically significant differences in the caloric value of the flatbreads, those with the addition of control yeast C1 had the highest caloric value, and the flatbreads with yeast enriched with iron using PEF had the lowest value.

Grain products, apart from fruit and vegetables, are the basis of the human diet. Flatbread is the oldest form of food that is still widely consumed in the Middle East, and due to the composition and methods of preparation, several varieties are distinguished, e.g., chapatti, lavash or tortillas [19]. The glycemic index (GI) is an index of foods that contain carbohydrates. It classifies foods based on their postprandial glycemic response against a reference carbohydrate source (glucose or white bread). It ranges from 1–100 [20]. The IG value depends on the size of starch molecules and the ratio of amylose to amylopectin, as well as the content of protein, fat, fiber, anti-nutrients, and organic acids [21]. The

investigated flatbreads did not differ significantly in IG and the values obtained in our study were mainly influenced by the type of flour used (refined wheat flour). However, these GI values were lower than those reported in the literature for traditional Indian flatbread [22] and can be classified as a product with a moderate GI.

2.2. Color Measurements

Table 2 shows the color of the flatbread surfaces in terms of L*, a*, b*, and ΔE values. The higher content of iron ions in the sample resulted in a decrease in brightness (L*) and yellowness (b*), increased redness (a*), thus an increase in the ΔE value for the sample with yeast P. This is the expected effect as the color of the baked goods depends on the color of the raw materials used. The iron-enriched yeast (C2 and P) were clearly darker than the unenriched yeast (C1). However, changing the color of iron-containing products may increase their acceptance, since darker products are perceived by consumers as healthier and associated with a higher content of health-promoting ingredients, e.g., dietary fiber [23].

Table 2. Color determinants of flatbread.

Flatbread with Yeast	L*	a*	b*	ΔE
C1	94.98 ± 0.54^{c}	1.00 ± 0.18^{a}	11.32 ± 0.52^{b}	-
C2	93.29 ± 0.22^{b}	1.54 ± 0.07^{c}	12.86 ± 0.14^{c}	3.78
P	91.53 ± 0.95^{a}	1.23 ± 0.1^{b}	10.47 ± 0.1^{a}	4.43

C1—yeast without the addition of iron ions and without PEF; C2—yeast with the addition of iron ions and without PEF; P—yeast with the addition of iron ions and PEF. Each value is the mean ± standard deviation ($n = 3$). Results with the same letter within a column are not significantly different ($p < 0.05$).

2.3. The Potential Bioavailability of Iron

Bioavailability is defined as the ability of a nutrient to be released from the food matrix and dissolved. It determines the amount of the active substance that enters the systemic circulation from the administered dose, as well as the rate of absorption of this substance. Many factors influence the bioavailability of a substance. It largely depends on the disruption of the permeability of the natural or processed food matrix, which in turn leads to the release of the nutrient that is absorbed in the gastrointestinal tract [24]. Iron absorption inhibitors are, for example, phytates, i.e., salts of phytic acid found in plants; polyphenols present in vegetables, fruits, some grains and legumes, tea, coffee, and wine. Calcium has also been shown to negatively affect non-heme and heme iron absorption, differentiating it from other inhibitors that only affect non-heme iron absorption [25]. One of the ingredients that improves iron absorption is ascorbic acid. This effect is largely due to its ability to reduce iron (III) to iron (II) as well as its ability to chelate iron [26]. In our study, refined wheat flour was used to produce the flatbread. The study did not analyze the content of phytate in flour and its influence on the bioavailability of iron. However, literature data show that similar flours are characterized by a phytate concentration of ≈100 mg/100g of flour. The effect of phytic acid on the bioavailability of non-heme iron is well known [27]. Since the same flour was used to obtain all the flatbreads and in the same proportion, the effect of phytates on the bioavailability of iron was ignored.

Table 3 presents the iron content and potential bioavailability of this metal from flatbreads. The flatbread obtained with the addition of unenriched yeast contained only about 3 mg/100 g dry mass of iron, and that with the enriched yeast, but without PEF, obtained about 266 mg/100 g of dry mass. The use of iron-enriched yeast in PEF conditions for the preparation of dough increased the iron content in the flatbreads to almost 386 mg/100 g dry mass. There was a significant difference in the potential bioavailability of iron between the samples. The flatbread with yeast P had the highest iron bioavailability, which was correlated with the highest content of this element in the product.

Table 3. Iron content and potential bioavailability of iron from flatbread.

Flatbread with Yeast	Iron Content (mg) in 100 g of Dry Mass	The Potential Bioavailability of Iron (%)
C1	2.96 ± 0.54 [a]	5.86 ± 0.12 [a]
C2	266.3 ± 2.62 [b]	7.97 ± 0.64 [b]
P	385.8 ± 4.12 [c]	10.83 ± 0.94 [c]

C1—yeast without the addition of iron ions and without PEF; C2—yeast with the addition of iron ions and without PEF; P—yeast with the addition of iron ions and PEF. Each value is the mean ± standard deviation (n = 3). Results with the same letter within a column are not significantly different ($p < 0.05$).

Many studies have been done to evaluate the iron bioavailability of cereal products fortified with iron [28]. However, it is difficult to compare the results due to differences in research methodology. For example, Pizarro et al. [26] prepared bread enriched with ferric sulphate which contained 47 mg of this element in 1 kg. The authors reported that the average iron absorption from this product determined in vivo was 10.5%, which is comparable to our results. There is a lack of reports using iron-enriched yeast for preparation of cereal products. In a study by Sabatier et al. [29], iron-enriched yeast were used for fortification of cheese. The authors determined the bioavailability of iron, but they used an in vivo method so it is difficult to compare the results. However, they concluded that iron from iron-enriched yeast was 72–82% as well absorbed as ferrous sulfate. Additionally, this study showed that during gastric and intestinal digestion in vitro, yeasts are lysed and release most of the iron after 1–3 h of the process. In our previous studies, we have shown that iron ions are bound by functional groups present both in the cell wall and in the intracellular structures of yeast [30].

2.4. Antioxidant Activity of Flatbread

Measuring the antioxidant properties of food products provides information about the antioxidant activity of a given product that may occur in the human body. The antioxidant activity of the flatbread was tested by the ability of the extracts to inhibit $DPPH^{\bullet}$ and $ABTS^{\bullet+}$. The investigated properties are presented in Figure 1. In the case of antioxidant activity against $DPPH^{\bullet}$ no significant differences were found between the samples. The highest values of antiradical activity against $ABTS^{\bullet+}$ were determined in flatbread with yeast C1, and the lowest in flatbread with yeast P (1.09 and 0.77 mMTE, respectively). The lower antioxidant capacity of the extract from flatbread with yeast P may result from the nature of iron, as iron is a metal with redox activity that can participate in electron transfer reactions, which in turn causes the production of oxidants capable of oxidizing cell components. Iron can participate in the catalysis of the formation of highly reactive hydroxyl radicals from hydrogen peroxide (H_2O_2) in the Haber-Weiss reaction and decompose lipid peroxides into peroxy and alkoxy radicals, which promotes lipid oxidation [31].

2.5. Sensory Evaluation

The results of the quality assessment of the flatbread using the 5-point rating scale are presented in Table 4. The quality of all the flatbreads was rated good and flatbread produced with yeast P obtained the highest average score. What is important, the panelist did not perceive a metallic after-taste which is a serious problem for products enriched with iron salts. The accumulation of iron in yeast may reduce unfavorable changes in taste in food products, since metal ions are associated with the cell organelles [30].

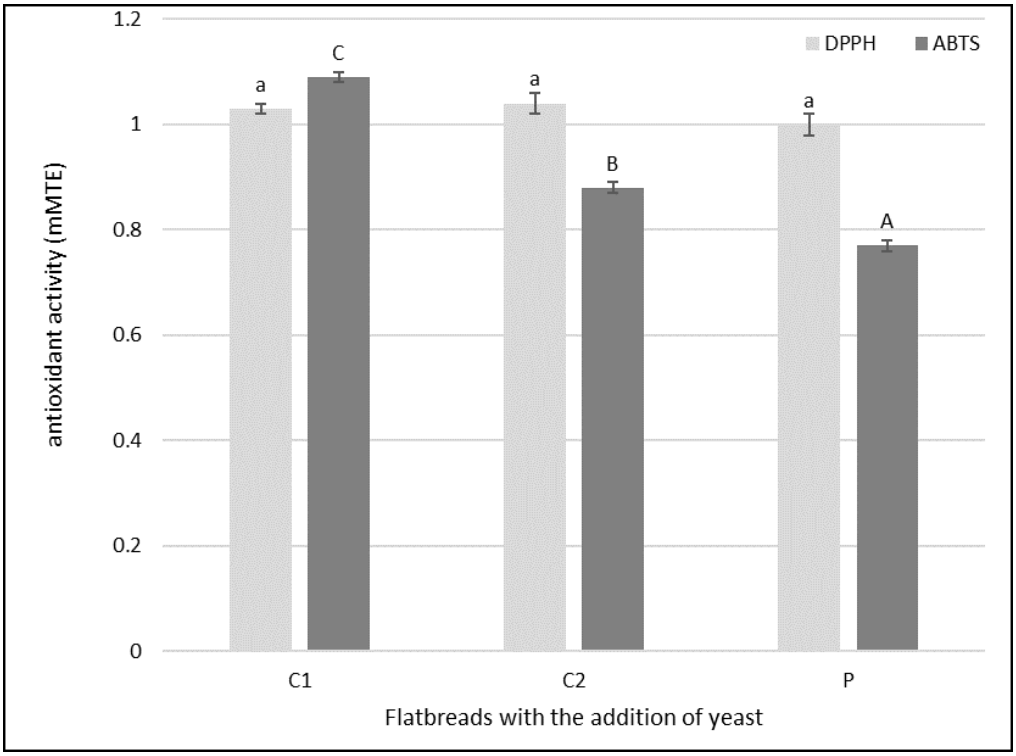

Figure 1. Antioxidant properties of flatbread produced with yeast not enriched with iron ions (C1), with yeast enriched with iron ions without PEF(C2), and with yeast enriched with iron ions using PEF(P), expressed as Trolox equivalent antioxidant activity. Each value is the mean ± standard deviation (n = 3). Bars with the same letter are not significantly different ($p < 0.05$).

Table 4. Results of the flatbread evaluation with the 5-point rating scale.

Feature	Weighting Factor	Flatbread with Yeast		
		C1	C2	P
Color	0.3	1.32 ± 0.10 ^{b}	1.22 ± 0.04 ^{a}	1.32 ± 0.03 ^{b}
Smell	0.15	0.39 ± 0.01 ^{b}	0.37 ± 0.02 ^{b}	0.33 ± 0.01 ^{a}
Structure and consistency	0.15	0.50 ± 0.08 ^{a}	0.56 ± 0.06 ^{a}	0.60 ± 0.02 ^{a}
Taste	0.4	1.55 ± 0.04 ^{a}	1.49 ± 0.08 ^{a}	1.68 ± 0.08 ^{b}
Overall		3.76 ± 0.12 ^{a}	3.64 ± 0.11 ^{a}	3.93 ± 0.03 ^{b}

C1—yeast without the addition of iron ions and without PEF; C2—yeast with the addition of iron ions and without PEF; P—yeast with the addition of iron ions and PEF. Each value is the mean ± standard deviation (n = 3). Results with the same letter within a row are not significantly different ($p < 0.05$).

The consumer's acceptance of the food products depends mainly on their sensory characteristics and health promoting properties. Due to the growing awareness of a healthy lifestyle, the importance of cereal products that contain whole grain or other functional ingredients is also increasing [18].

The use of iron-enriched yeast as a supplement is becoming more and more popular [32–34]. However, there are still few reports of the use of iron-enriched yeast in food products.

Iron compounds that are characterized by poor solubility at normal gastric acid concentrations do not interfere with the sensory properties of food [35]. On the other hand, more soluble compounds can cause fat oxidation (i.e., going rancid) as well as a color change in the product within 6 months of storage. Reduced iron is preferred, and the smaller the particle size, the better it will be absorbed [36]. According to Kiskini et al. [37], who compared sensory characteristics of unenriched bread with breads that were enriched with various iron compounds, ferric pyrophosphate was the compound that after addition to bread gave the most acceptable product, while bread with ferrous lactate was the least

acceptable. However, all the products had a pungent odor that was not found in the iron-enriched flatbread obtained in this study.

The sensations of smell and taste consist of the basic smell and taste derived from the aromatic compounds of the basic ingredients and aromatic additives in the composition of the raw materials. The smell of a product will meet the required standards if it is characteristic for a given product and will be free of foreign, unusual smells [38]. According to the established average values of the smell of the flatbread, the highest score of 0.39 was given to the flatbread with yeast C1 (unenriched with iron), but the differences between the scores for all the flatbreads were very small.

Appearance is based on the sense of sight and includes an ability that can be examined visually. Consumers often decide whether to buy a given product based on the appearance of the product. Color also affects the appearance. This is an important feature as it is used as a control parameter when baking wheat flour products [38]. It can be concluded from Table 4 that the color of the flatbread with the addition of C1 and P yeast was ranked the highest. The color of the flatbread with the addition of P yeast resembled whole grain products, which, due to the growing awareness of consumers about a healthy lifestyle, are more acceptable and desirable.

The flatbreads with the addition of yeast P obtained the highest scores for structure and consistency. The quality of flatbread is primarily influenced by the type of flour used and the total protein content in the flour. Many of the quality characteristics, e.g., external appearance, structure, and consistency, depend on the quality and quantity of the protein. In leavened bread, the higher water absorption results in more carbon dioxide bubbles and a coarser structure of the bread. The water used when mixing the ingredients allows the formation of gluten as a result of protein hydration and the change of rheological properties [18].

2.6. Limitations and Future Perspectives

The results obtained in this study suggest that the use of yeast enriched with iron through its accumulation enhanced by PEF is a promising tool for the production of functional foods that may be effective against, e.g., anemia. The main limitation of the use of PEF to enrich yeast with bio-elements, including iron, is that despite many scientific studies on the principles and applications of PEF published so far, this technology is still considered emerging. There are still no specific regulations in the European Union for food processed with PEF. Moreover, consumers are suspicious of food produced with the use of unconventional methods. What is more, the use of such methods entails additional costs. Therefore, at present, the fortification of food with iron-enriched yeast cannot yet compete with the fortification with iron salts in this respect.

3. Materials and Methods

3.1. Ingredients for the Production of Flatbread

Flatbread was prepared in three trials with the addition of yeast not enriched with iron ions (C1), yeast enriched with iron ions (C2) by a supplementation of medium, and yeast enriched with iron ions using a pulsed electric field (P). Additionally, 450 g of wheat flour (white wheat flour for pastry, type 450), 7 g of freeze-dried yeast (the composition of yeast is given in Table 5), salt, olive oil, and 30 mL of warm water were used to prepare the dough.

3.2. Yeast Strain and Culture Conditions

In the experiment, the yeast strain *Saccharomyces cerevisiae* 11 B1 from the Department of Biotechnology, Microbiology and Human Nutrition of the University of Life Sciences in Lublin, was used. Then, agar slants and inoculum were prepared according to Romani and Maguire [39].

Table 5. Composition of wheat flour and freeze-dried yeast used for the production of flatbread (content given on a dry mass).

Component	Protein (%)	Carbohydrates (%)	Fat (%)	Iron Content (mg/g)
Wheat flour	10.8 ± 0.21 a	65.7 ± 0.18 c	1.35 ± 0.12 a	0.70 ± 0.01 a
Yeast C1	59.13 ± 0.18 b	31.79 ± 0.28 a	2.7 ± 0.14 b	0.13 ± 0.01 b
Yeast C2	58.24 ± 0.35 c	30.44 ± 0.59 b	2.5 ± 0.00 c	18.68 ± 0.86 c
Yeast P	54.0 ± 0.11 d	31.00 ± 0.37 b	2.35 ± 0.07 d	48.01 ± 0.88 d

C1—yeast without the addition of iron ions and without PEF; C2—yeast with the addition of iron ions and without PEF; P—yeast with the addition of iron ions and PEF. Each value is the mean ± standard deviation (n = 3). Results with the same letter within a column are not significantly different ($p < 0.05$).

3.3. Preparation of Yeast Culture

Ten milliliters of the inoculum was added to 80 mL (C2, P) or 90 mL (C1) of the culture medium in a 500 mL Erlenmeyer flask. The cultivation was carried out under the same conditions as the inoculum. To each flask (except sample C1), 10 mL of iron (III) nitrate solution was added so that the final concentration of iron ions in the medium was 200 µg Fe^{2+}/mL. The culture was then incubated at 30 °C for 20 h. The culture P was exposed to PEF for 20 min at a pulse width of 10 µs, an electric field voltage of 1500 V, at a field frequency of 1 Hz using a laboratory electroporator (ECM 830, BTX Harvard Apparatus, Holliston, MA, USA)(except for control samples C1 and C2). Cultures were then incubated for 22 h. The biomass was centrifuged (10 min, 3000 rpm), the supernatant was discarded, and the cells were washed three times with deionized water [30] and freeze-dried (Labconco, Kansas City, MO, USA).

3.4. Preparation of Flatbread

Seven grams of yeast (C1, C2, P) was grown in warm water (30 mL) for 45 min at room temperature. Then, 450 g of flour, 1 g of salt, and 10 g of olive oil were added to the prepared mixture. The mixtures were prepared in the traditional way by weighing the ingredients according to the recipe, thorough mixing, and aeration. Afterwards, the dough was rolled out and fried in a hot pan on both sides for 2 min. At the same time, control samples were prepared for the flatbread containing yeast without the addition of iron ions (C1) and yeast with the addition of iron ions and not treated with PEF (C2). Each type of flatbread was produced in three repetitions.

3.5. Nutrient Composition and Energy Content

Flatbreads were analyzed for protein content by the Kjeldahl method (N × 6.25), fat, and ash using standard analyzes [40]. The carbohydrate content was calculated according to the formula: 100−(weight in grams (protein + fat + ash) in 100 g of dry weight of flatbreads.

The energy content of the products was determined by multiplying the values obtained for protein, available carbohydrates, and fat by 4.00, 4.00, and 9.00, respectively, and adding up the results [41].

3.6. Color Measurements

From each trial, 10 g of flat bread was randomly selected and the color was measured with an EnviSense NH310 colorimeter (EnviSense, Lublin, Poland) in triplicate. Color differences were recorded on the CIE L* a* b* scale with respect to brightness (L^*) and color (a^*—redness; b^*—yellow). The total color difference (ΔE) was calculated from the formula (1):

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (1)$$

where ΔL^*,Δa^*, and Δb^* are differences in the L^*, a^*, and b^* values, respectively, between the reference sample and the test sample.

3.7. Antioxidant Properties

3.7.1. Extraction of Bioactive Compounds

The samples (1 g) were ground in a laboratory grinder and shaken with 10 mL of 4:1 ethanol/water (*v*/*v*) for 120 min in a laboratory shaker. Next, the samples were centrifuged at 3000 g for 10 min. The supernatant was taken and stored at −18 °C.

3.7.2. DPPH Radical Scavenging Activity

The DPPH assay was performed according to Brand-Williams et al. [42] with modification. An aliquot of 0.1 mL of the sample was mixed with 0.9 mL of a 6 µM solution of DPPH$^{\bullet}$ in 75% methanol and left for 3 min. The absorbance at 515 nm was then measured against 75% methanol as a blank. The determination was performed in triplicate. The scavenging effect was calculated according to the formula (2):

$$\text{Scavenging activity } (\%) = \left[1 - \left(\frac{\text{A sample}}{\text{A control}}\right)\right] \times 100 \quad (2)$$

where the A sample is the absorbance of the mixture of sample and DPPH$^{\bullet}$ and the A control is the absorbance of the control (DPPH$^{\bullet}$ solution). The results were expressed as Trolox equivalent antioxidant activity (TEAC) values (mM Trolox).

3.7.3. ABTS Radical Scavenging Activity

The ABTS assay was performed according to Re et al. [43] with slight modifications. Here, 2.90 mL of the ABTS$^{\bullet+}$ solution was mixed with 0.1 mL of each sample. The absorbance was measured at 734 nm after 3 min of the reaction against deionized water. The scavenging effect was calculated using Equation (3):

$$\text{Scavenging activity } (\%) = \left[1 - \left(\frac{\text{A sample}}{\text{A control}}\right)\right] \times 100 \quad (3)$$

where the A sample is the absorbance of the mixture of sample and ABTS$^{\bullet+}$ and the A control is the absorbance of the control (ABTS$^{\bullet+}$ solution).The results were expressed as Trolox equivalent antioxidant activity (TEAC) values (mM Trolox).

3.8. Potential Bioavailability of Iron from Flatbread

The in vitro digestion was performed according to Szalast-Pietrzak et al. [44] with slight modifications. Here, 1 g of the sample was combined with 30 mL of deionized water and 1 M HCl to obtain a pH of 2.0 and treated with pepsin (Sigma-Aldrich, St. Louis, MO, USA) in the amount of 2 mL of a 10% enzyme solution in 0.1 M HCl per test system. The reaction was carried out for 75 min at 37 °C with stirring (130 rpm). The second step of the in vitro process, corresponding to intestinal digestion, was performed using dialysis tubes with a molecular weight cutoff of 14 kDa. In this digestion stage, the pH of the test systems was adjusted to pH 6.5 with 6% $NaHCO_3$ solution and treated with pancreatin (Sigma-Aldrich) in the amount of 5 mL of 0.4% enzyme solution in 0.1 M $NaHCO_3$ per test system. Then, the samples were quantitatively transferred to dialysis tubes, which, after being sealed, were placed in laboratory containers made of PP material in 500 mL of deionized water. After the intestinal digestion step, 3 mL of dialysate and dialysis solution were taken (Figure 2).

The iron content of the dialysate, the dialysis solution, and the product before digestion was determined by the FAAS method. The data were substituted into a formula (4) taken from Szalast-Pietrzak et al. [44] and the potential bioavailability of iron was calculated:

$$B\% = [D + Dr/(T + D)] \times 100\% \quad (4)$$

where D is the amount of iron (mg) in the dialysate, Dr is the amount of iron (mg) contained in the tube dialysis treatment corresponding to the balance of concentrations, and T is the amount of iron (mg) in the tube (mineralizate).

The amount of iron (mg) in the dialysis tube corresponding to the equilibrium concentrations of the given test system was calculated according to the formula (5) taken from Szalast-Pietrzak et al. [44]:

$$Dr = (D \times Vt)/Vd \quad (5)$$

where D is the amount of iron (mg) in the dialysate, Vt is the sample volume in the tube (mL), and Vd is the dialysate volume (mL).

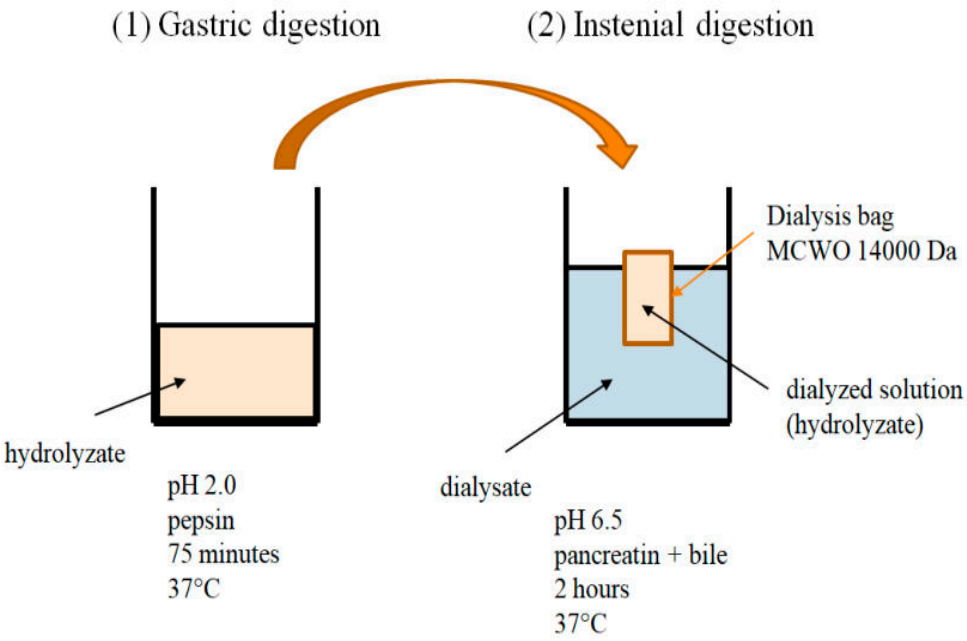

Figure 2. Scheme of in vitro digestion: (**1**) gastric digestion: enzyme: pepsin, pH: 2.0, digestion time: 75 minutes at 37 °C; (**2**) intestinal digestion: enzymes: pancreatin and bile, pH: 6.5, digestive time: 2 hours in 37 °C.

3.9. In Vitro Glycemic Index (GI)

The glycemic index (GI) of flatbread was determined according to the method of Reis and Abu-Ghannam [45] with slight modifications. The digestion procedure outlined in Section 3.8 was used. Here, 1 mL of the hydrolyzate was collected during the in vitro digestion period at 10, 20, 30, 60, 90, 120, and 180 min of digestion. Then, 4 mL of ethanol was added to 1 mL of the hydrolyzate to deactivate the enzymes. The glucose content in the hydrolysates was determined using the GOPOD method. Values are expressed as mg glucose/g sample. The glucose content was plotted as a function of time and the areas under the hydrolysis curves (AUC) were calculated. The hydrolysis index (HI) for each sample was calculated as the ratio between the AUC of the sample and the AUC of the reference food, which was white bread. The value was expressed as a percentage. The GI was calculated according to the Equation (6) described by Goñi, Garcia-Alonso and Saura-Calixto [46]:

$$GI\ (\%) = 39.71 + 0.549 \times HI \quad (6)$$

3.10. Sensory Evaluation

A group of 15 trained people aged from 28 to 50 participated in the sensory test using a five-point rating scale with definitions for each point value on the scale. People participating in the evaluation received previously prepared evaluation cards (Table 6). The evaluation of qualitative factors such as: color, smell, structure and consistency, as

well as taste was made on the basis of the developed scheme (Table 7). The individual qualitative characteristics were assigned a weighting factor. A grade of 5 meant a very good class, 4—a good class, 3—a satisfactory class, 2—an insufficient class, and 1—a bad class. The samples were codded with three-digit numbers and served on white plates. The assessments were made in the correct order. First, the visual characteristics were assessed, then the rheological ones, and the last was the taste of the product.

Table 6. Evaluation card for the 5-point rating scale.

Feature	Sample No.		
	218	331	829
Color			
Smell			
Structure and consistency			
Taste			

Table 7. Scheme of the 5-point rating scale.

Feature	Weighting Factor	Definitions				
		5	4	3	2	1
Color	0.30	Light with brown baked bubbles	Light with little-baked bubbles	Bright, with traces of baked bubbles	No characteristic baked bubbles	Inappropriate color—very light or very dark
Smell	0.15	Very aromatic, typical for a baked flour product	Aromatic, typical for a baked flour product	A noticeable smell of a baked flour product	Faint smell of baked flour product, perceptible smell of a burnt product	No smell characteristic of flour products, a very perceptible smell of a burnt product, foreign smell
Structure and consistency	0.15	Very well baked, compact, uniform, easily brittle	Well baked, brittle	Sufficiently baked and hard, brittle	Poorly baked, not too hard, not brittle	Underdone, rubbery
Taste	0.40	Very natural, mild, character-isticfor flour product	Natural, gentle, desirable	Sufficiently natural, mild, without any foreign taste	Very poor taste of flour products, not very natural	No flour taste, bitter taste, metallic after-taste

3.11. Statistical Analysis

Regression analyses and significance tests were performed using the Statistica version 13.3 software (StatSoft, Inc., Tulsa, OK, USA). The post-hoc Tukey test was employed to determine differences between means. Results of $p < 0.05$ were considered statistically significant.

4. Conclusions

Micronutrient malnutrition is a major contributor to the increase in the incidence of various diseases. Iron deficiency is a common cause of anemia that affects people of all ages and around the world. Food fortification with iron compounds is one of the strategies for its prevention, but unfortunately iron can cause unacceptable sensory changes in products. In our study, rather than adding iron compounds directly to the product, we used yeast enriched with iron ions to produce flatbread. We applied pulsed electric field to enhance iron accumulation in yeast cells. The obtained flatbread contained about 386 mg/100 g dry mass of iron, had good potential bioavailability of this element, was acceptable to consumers (no metallic aftertaste was detected), and had a moderate glycemic index.

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ORIGINAL ARTICLE

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Preparation of yeast flakes enriched with iron and vitamin B_{12} using a pulsed electric field technology

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Abstract

In this study pulsed electric field (PEF) was used to increase accumulation of iron and vitamin B_{12} in *Saccharomyces cerevisiae* cells, which were then used to produce flakes. The yeast was enriched in two ways: only by adding iron salt ($FeCl_3 \cdot 6H_2O$) and vitamin B_{12} to the culture medium and by additional application of PEF. Three trials of yeast flakes were prepared: C1 (control, not supplemented), C2—with B_{12} and iron salt and not treated with PEF, and P—with B_{12} and iron salt and treated with PEF (for 20 min at 10 μs pulse width, voltage of 1500 V, a number of pulses 1200 and field frequency 1 Hz). The use of PEF resulted in higher iron and vitamin B_{12} accumulation, respectively, 2.5- and 1.4-fold for the sample P compared to the sample C2. The potential bioavailability of iron from yeast flakes was 10.13% and vitamin B_{12}–4.31%, and the quality of this product was rated good by a sensory panel.

KEYWORDS
deficiency, iron, pulsed electric filed, vitamin B_{12}, yeast

1 | INTRODUCTION

The rise in popularity of vegetarian and vegan diets has been observed worldwide and across age groups. However, eliminating all animal products from the diet increases the risk of nutritional deficiencies. Micronutrients that are particularly important for vegans and vegetarians include vitamin B_{12} and iron in particular. Therefore, in case of deficiencies of these components, supplementation should be considered and appropriate eating habits implemented (Weikert et al., 2020).

Iron in plant foods is in the nonheme form, which may be absorbed to a lesser extent than the heme iron found in meat, fish, and their meat products (bioavailability 1%–34% and 15%–35%, respectively). Dietary factors and cooking techniques affect the absorption of nonheme iron. Factors that increase the bioavailability of iron are the presence of ascorbic acid (vitamin C), malic acid and low pH. Greater iron absorption occurs from fermented foods due to low pH and the presence of lactic acid. The effect of reducing iron absorption is shown by dietary fiber, phytates, which are found in cereals and legumes, polyphenols, high amounts of calcium, and zinc (Baroni et al., 2019).

Long-term iron deficiency results in anemia that is the cause of decreased work productivity, increased child and maternal mortality, and slowed child development. Mild and moderate anemia may increase susceptibility to infectious diseases (Govindappagari & Burwick, 2019).

Vitamin B_{12} (cobalamin) is a water-soluble vitamin. Its sources include fish, meat and dairy products, as well as fortified cereal products and supplements. This vitamin is crucial for neurological function and is involved in red blood cell production and DNA synthesis (Langan & Goodbred, 2017).

One way to treat iron and vitamin B_{12} deficiency is oral supplementation. However, the use of dietary supplements is associated with side effects such as gastrointestinal complaints. Additionally, dietary supplements are characterized by low bioavailability of iron (Shubham et al., 2020) and vitamin B_{12} (O'Leary & Samman, 2010). Mineral and vitamin deficiencies can be prevented or treated by food fortification. Yeast can be a good source of minerals and vitamins in a diet and it can be modified by appropriate supplementation of the nutrient medium. Products containing *Saccharomyces cerevisiae* are widely available in tablet, powder, flake, and liquid forms. For flakes production, yeast can be cultured on molasses enriched with

additional nutrients such as minerals and B vitamins under aerobic conditions; the nutrients in the resulting product will depend on the composition of the molasses (Bekatorou et al., 2006). The flakes prepared in this way, for example, can be used as a flavor enhancer and source of umami flavor compounds (Grasso et al., 2019). This product may also be in the form of a yellow powder with a consistency similar to corn flour (Satyanarayana & Kunze, 2017).

Accumulation of mineral nutrients in yeast cells can be enhanced by treatment with pulsed electric field (PEF) (Nowosad, Sujka, Pankiewicz, Miklavčič, et al., 2021; Pankiewicz et al., 2017). This application of PEF is unique because usually this technology is used in yeast research to inactivate cells (Cserhalmi et al., 2002) or to increase the extraction of intracellular compounds (Dimopoulos et al., 2018). The action of PEF is based on the phenomenon of electroporation of the cell membrane. Electroporation involves the formation of pores in lipid membranes. It has been hypothesized that small molecules and ions can pass through the pores. In this way, for example, in yeast cells, metal-protein complexes called metalloproteins (or bioplexes) are formed, which are highly bioavailable to the human body (Nowosad, Sujka, Pankiewicz, & Kowalski, 2021). The effect of PEF on yeast depends, among others, on the PEF protocol and yeast growth phase (Mattar et al., 2014).

As previous research has shown, application of PEF increases the accumulation of minerals in *S. cerevisiae* cells, for example, zinc (Pankiewicz & Jamroz, 2011), selenium (Pankiewicz et al., 2017), magnesium (Pankiewicz & Jamroz, 2010) and iron (Nowosad, Sujka, Pankiewicz, Miklavčič, & Arczewska, 2021). In the studies by Pankiewicz and Jamroz (2010, 2011), the accumulation of magnesium and zinc in cells increased by 40% and 63%, respectively, after the PEF treatment. Research has not yet been conducted on the use of PEF to increase vitamin accumulation in yeast cells. However, enrichment of yeast cultures by addition of vitamins to the medium has already been investigated, for example, Jach et al. (2020) obtained biomass of unconventional *Yarrowia lipolytica* A-101 yeast supplemented with vitamin B_{12} (9 μg of vitamin B_{12} per 100 g of dry biomass).

In this study we produced yeast flakes using yeast enriched with iron ions and vitamin B_{12} by two methods: only by adding iron salts and vitamin B_{12} to the medium and by additional increasing the accumulation by the PEF action. Our main objective was to investigate the potential bioavailability of iron and vitamin B_{12} from yeast flakes prepared in this way and to investigate the nutritional and antioxidant properties of this food product. In addition, we investigated the effect of pulsed electric field on vitamin B_{12} accumulation in *Saccharomyces cerevisiae* cells.

2 | MATERIALS AND METHODS

2.1 | Reagents and microorganisms

All reagents used were of analytical grade purity. Vitamin B_{12}, DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), iron (III) chloride, pepsin and pancreatin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Yeast strain *Saccharomyces cerevisiae* 11 B1 was obtained from the Department of Biotechnology, Microbiology and Human Nutrition at University of Life Sciences in Lublin. Agar slants and inoculum were prepared according to Romani and Maguire (2002). The composition of medium for agar slants and inoculum growth, as well as that used in the experiment with PEF was presented by Nowosad, Sujka, Pankiewicz, Miklavčič, et al. (2021).

2.2 | Yeast culturing conditions and PEF protocol

Five yeast cultures (Y1–Y5) with the composition presented in Table 1 were prepared in 500 ml Erlenmeyer flasks. The cultivation was carried out according to the procedure by Romani and Maguire (2002). The total cultivation time was 40 h and the final concentration of iron ions in the medium was 200 μg Fe^{3+}/ml, and vitamin B_{12}–1 mg/ml. The cultures Y4 and Y5 were subjected to PEF using an ECM 830 unipolar square wave generator (BTX Harvard Apparatus, Holliston, MA, USA) after 20 h of cultivation according to the procedure described by Nowosad, Sujka, Pankiewicz, Miklavčič, et al. (2021). PEF parameters were as follows: voltage of 1500 V (the electric field strength of 3 kV/cm), pulse width of 10 μs, treatment time 20 min, number of pulses 1200 and field frequency 1 Hz. For these cultures, both the ferric ion solution and the vitamin B_{12} solution were added just prior to electroporation. Then all PEF-treated samples were incubated in an incubator with shaking of 100 rpm at 30°C for next 20 h. After 40 h cultivation all samples were centrifuged and washed several times with deionized water.

2.3 | Selection of vitamin B_{12} concentration

Freshly prepared vitamin B_{12} solution was added to the medium so that the concentration of vitamin B_{12} in the sample was 0.25, 0.5, and 1 mg/ml, respectively. Subsequently, the culture was subjected to PEF with optimal parameters for iron accumulation described in the

TABLE 1 Composition of the samples used in the experiment

Yeast	Medium (ml)	Inoculum (ml)	Ferric ions solution (ml)	Vitamin B_{12} solution (ml)	PEF treatment
Y1 (control)	90	10	–	–	–
Y2	80	10	–	10	–
Y3	70	10	10	10	–
Y4	70	10	–	10	+
Y5	70	10	10	10	+

Abbreviation: PEF, pulsed electric field.

Section 2.2. The control sample Y1 (not supplemented with iron and B_{12}, no PEF treatment) was prepared at the same time. The vitamin B_{12} content in the samples was determined by the HPLC method described in Section 2.8. The iron content was simultaneously determined in the yeast samples Y1–Y5 by the flame atomic absorption spectrometry (FAAS) (see Section 2.8).

2.4 | Preparation of yeast flakes

Yeast from the cultures Y1 (not enriched with iron ions and vitamin B_{12}, not treated with PEF), Y3 (enriched with ferric ions and vitamin B_{12} only by supplementing the nutrient medium) and Y5 (enriched with ferric ions and vitamin B_{12} by supplementing the nutrient medium and application of PEF) were used for yeast flakes preparation (samples C1, C2, P, respectively). After centrifugation of each culture, the yeast samples were transferred to a laboratory dryer and dried on the Petri dishes at 50°C for 1.5 h until the samples have a water content typical for the commercial flakes. After this step, a portion of each sample was used immediately for the determination of vitamin B12 content, and the rest was transferred to plastic containers and stored at 4°C overnight.

2.5 | Nutritional composition and energy content

Yeast and/or yeast flakes were analyzed for protein content by Kjeldahl method (N × 5.8), fat and ash using standard analyzes (AOAC International, 2010). The carbohydrate content (%) was calculated according to the formula:

$$\text{Carbohydrate content}(\%) = 100\,\text{g} - [(\text{weight in grams}(\text{protein} + \text{fat} + \text{ash})\ \text{in 100 g of dry weight})] \quad (1)$$

The energy content of the flakes was determined by multiplying the values obtained for protein, digestible carbohydrates and fat by 4.00, 4.00 and 9.00, respectively, and summing the results.

2.6 | Color measurements

Color of randomly selected portion (10 g) of each sample was measured with the EnviSense NH310 colorimeter (EnviSense, Lublin, Poland). Color differences were recorded on the CIE $L^*a^*b^*$ scale with respect to brightness (L^*) and color (a^* - redness; b* - yellow). The device was calibrated on a white standard before testing. The temperature of the test was equal to the ambient temperature and it was 20°C. The total color difference (ΔE) was calculated from the formula:

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}} \quad (2)$$

where ΔL^*, Δa^*, and Δb^* are differences in the L^*, a^*, and b^* values, respectively, between the reference sample and the test sample.

2.7 | Antioxidant activity

2.7.1 | Extraction of bioactive compounds

Samples (1 g) were ground in a lab grinder and shaken with 10 ml of 4:1 ethanol/water (v/v) mixture for 120 min on a lab shaker (Paramera et al., 2011). The samples were then centrifuged at 3000 *g* for 10 min. The supernatant was removed and stored at −18°C.

2.7.2 | ABTS radical scavenging activity

The ABTS assay was performed according to Re et al. (1999) with minor modifications. Here, 2.90 ml of $ABTS^{\bullet+}$ solution was mixed with 0.1 ml of each sample. Absorbance was measured at 734 nm after a 3 min reaction with deionized water. The scavenging effect was calculated using the equation:

$$\text{Scavenging activity}\,(\%) = \left[1 - \left(\frac{A\text{sample}}{A\text{control}}\right)\right] \times 100 \quad (3)$$

where: A_{sample} is the absorbance of the mixture of sample and $ABTS^{\bullet+}$ and $A_{control}$ is the absorbance of the control ($ABTS^{\bullet+}$ solution).The results were expressed as Trolox equivalent antioxidant activity (TEAC) values (mM Trolox).

2.7.3 | DPPH radical scavenging activity

The DPPH assay was performed according to Brand-Williams et al. (1995) with slight modification. An aliquot of 0.1 ml of the sample was mixed with 0.9 ml of a 6 μM solution of $DPPH^{\bullet}$ in 75% methanol and left for 3 min. The absorbance at 515 nm was then measured against 75% methanol as a blank. The scavenging effect was calculated according to the formula:

$$\text{Scavenging activity}\,(\%) = \left[1 - \left(\frac{A\,\text{sample}}{A\,\text{control}}\right)\right] \times 100 \quad (4)$$

where: A_{sample} is the absorbance of the mixture of sample and $DPPH^{\bullet}$ and $A_{control}$ is the absorbance of the control ($DPPH^{\bullet}$ solution). The results were expressed as Trolox equivalent antioxidant activity (TEAC) values (mM Trolox).

2.8 | Potential bioavailability of iron and vitamin B_{12} from yeast flakes

The in vitro digestion was performed according to Szalast-Pietrzak et al. (2018) with slight modifications described by Nowosad and Sujka (2021). Here, 1 g of the sample was combined with 30 ml of deionized water and 1 M HCl to obtain a pH of 2.0 and treated with pepsin in the amount of 2 ml of a 10% enzyme solution in 0.1 M HCl per test system. The reaction was carried out for 75 min at 37°C with

stirring (130 rpm). The second step of the in vitro process, corresponding to intestinal digestion, was performed using dialysis tubes with a molecular weight cutoff of 14 kDa. In this digestion stage, the pH of the test systems was adjusted to pH 6.5 with 6% $NaHCO_3$ solution and treated with pancreatin in the amount of 5 ml of 0.4% enzyme solution in 0.1 M $NaHCO_3$ per test system. Then, the samples were quantitatively transferred to dialysis tubes, which, after being sealed, were placed in laboratory plastic containers in 500 ml of deionized water. After the intestinal digestion step, 3 ml of dialysate and dialysis solution were taken.

The iron content of the dialysate, the dialysis solution, and the product before digestion was determined by the FAAS method (Nowosad, Sujka, Pankiewicz, Miklavčič, et al., 2021). For determination of B_{12} content samples were filtered and analyzed by gradient HPLC system (Beckman, System GOLD, USA) equipped with an ion exchange column (Gemini 3µ C18 110A, 150 × 4.6 mm 3 µm), Phenomenex with a pre-column Gemini (4,3 mm, C18, Phenomenex, USA), diode array detector (type 166), and gradient solvent module pomp (type 126AA), a dosing valve 7725i (Rheodyne, USA) and a column thermostat. The mobile phase consisted of solvent A (0.6% acetic acid) and solvent B (80% acetonitrile) with a flow rate of 1.0 ml/min. The initial mobile phase composition was maintained at 100% solvent changed linearly to 95% (0–5 min), then changed linearly to 65% (5–22,5 min) and held 5,5 min (22,5–28 min), then followed by a return to the initial conditions within 3 min (28–31 min) and kept 5 min (31–36 min) for the chromatograph column equilibrium. Chromatograms were recorded at 361 nm. The concentration of wit B_{12} was calculated from equation of the calibration curve plotted for the standard solutions. Gold Nouveau Chromatography Data System, version 1.7 (Beckman, USA) was used for process control and data collection. The injection volume was 20 µl.

The data were substituted into the following formula (Szalast-Pietrzak et al., 2018) and the potential bioavailability of iron or vitamin B_{12} was calculated:

$$B\% = [D + D_r/(T + D)] \times 100\% \quad (5)$$

where: D is the amount of iron (mg) or vitamin B_{12} (µg) in the dialysate, D_r is the amount of iron (mg) or vitamin B_{12} (µg) contained in the tube dialysis treatment corresponding to the balance of concentrations, and T is the amount of iron (mg) or vitamin B_{12} (µg) in the tube.

The amount of iron (mg) and vitamin B_{12} (µg) in the dialysis tube corresponding to the equilibrium concentrations for of the given test system was calculated according to the formula (Szalast-Pietrzak et al., 2018):

$$D_r = (D \times V_t)/V_d \quad (6)$$

where: D_r, amount of iron (mg) or vitamin B_{12} (µg) in the dialysate, V_t, sample volume in the tube (ml), V_d, dialysate volume (ml).

2.9 | In vitro glycemic index

The glycemic index (GI) of yeast flakes was determined according to the method of Reis and Abu-Ghannam (2014) with slight modifications. The digestion procedure outlined in the previous section was used. Here, 1 ml of the hydrolysate was collected during the in vitro digestion period at 10, 20, 30, 60, 90, 120, and 180 min of digestion. Then, 4 ml of ethanol was added to 1 ml of the hydrolysate to deactivate the enzymes. The glucose content in the hydrolysates was determined using the glucose oxidase/peroxidase assay (GOPOD method, Megazyme). Values are expressed as mg glucose/g sample. The glucose content was plotted as a function of time and the areas under the hydrolysis curves (AUC) were calculated. The hydrolysis index (HI) for each sample was calculated as the ratio between the AUC of the sample and the AUC of the reference food, which was white bread. The value was expressed as a percentage. The GI was calculated according to the equation described by Goñi et al. (1997):

$$GI\,(\%) = 39.71 + 0.549 \times HI \quad (7)$$

2.10 | Sensory evaluation

A group of 15 trained people aged from 18 to 60 participated in the sensory test using a five-point rating scale with definitions for each point value on the scale. The evaluation of qualitative factors such as: color, smell, structure and consistency, and taste was made on the basis of the developed scheme (Table 2). Each quality feature was assigned a weighting factor. A grade of 5 meant a very good class, 4—a good class, 3—a satisfactory class, 2—an insufficient class, and 1—a bad class. The samples were codded with three-digit numbers and served on white plates. The assessments were made in the correct order: first, the visual characteristics were assessed, then the rheological ones, and the last was the taste of the product. Then, based on the individual results, an average was drawn and multiplied by the weighting factor assigned to the feature. The results were summed to provide an overall rating for product quality.

2.11 | Statistical analysis

All measurements were performed in at least three repetitions. Regression analyses and significance tests were performed using the Statistica version 13.3 software (StatSoft, Inc., Tulsa, OK, USA). The post-hoc Tukey test was used to determine differences between means. Results of $p < .05$ were considered statistically significant.

3 | RESULTS AND DISCUSSION

3.1 | Effect of vitamin B_{12} concentration on its accumulation in yeast

Accumulation of ions and other molecules in yeast cells depends on the concentration of molecules and ions and their bioavailability (Williams & Da Silva, 2000). In our experiment, the application of PEF increased the accumulation of vitamin B_{12} in all tested concentrations

TABLE 2 Scheme of the 5-point rating scale

Feature	Weighting factors	Definitions				
		5	4	3	2	1
Color	0.25	Solid color, no other shades visible	Solid color, with slight visible other shades	Solid color with a moderate amount of other shades	Color inconsistent over large areas of the surface	Wrong color, not solid over the entire surface
Smell	0.25	Very aromatic, characteristic for a yeast product	Aromatic, characteristic of a yeast product	Noticeable smell of the yeast product	Faint smell of yeast product	No smell characteristic of yeast products
Structure and consistency	0.15	Very well done, uniform, easily brittle and crumbly	Well done, brittle and crumbly	Sufficiently baked and firm, brittle	Poorly baked, not too hard, not brittle	Underdone, rubbery
Taste	0.35	Very natural, mild, characteristic yeast taste, without any foreign taste	Natural, mild, desirable, without any foreign taste	Sufficiently natural, mild, without any foreign taste	Very little taste of yeast products, not very natural, little metallic taste	No yeast taste, noticeable metallic taste

of this vitamin (0.25 mg/ml medium, 0.5 mg/ml medium and 1 mg/ml medium) (Figure 1). However, the greatest effect of PEF was observed for the concentration of 1 mg/ml of medium. The content of vitamin B_{12} in sample Y5 (yeast enriched with 1 mg vitamin B_{12}/ml medium and 200 μg Fe^{3+}/ml medium using PEF) was 50% higher than in sample Y3 (yeast enriched with the same concentration of both substances but without PEF). Therefore, we decided to use this concentration of vitamin B_{12} in the next stage of the experiment. Additionally, we observed that yeasts Y3 and Y5 accumulated more B_{12} than yeasts Y2 and Y4. The possible explanation is that the presence of ferric ions has a stabilizing effect on this vitamin (Heep & Taterra, 2015).

The accumulation of ions and vitamins by microorganisms occurs in several ways. Metal ions can accumulate on the surface of microbial cells in an undefined way and also due to the sorptive properties of microbial cells. A common phenomenon is also the active transport of some ions (e.g., magnesium, sodium, potassium) into the cells of microorganisms. In some cases, microorganisms secrete chelating compounds and ionophores that enable them to take up an ion (Stehlik-Tomas et al., 2004). In our research, PEF was used to increase the accumulation of vitamin B_{12} and iron in yeast cells. As a result of PEF action on a cell, a transmembrane potential difference is induced. According to the widely accepted theoretical model, hydrophilic pores are formed in the lipid bilayer of a cell membrane by spontaneous thermal fluctuations of membrane lipids. This leads to an increase in the permeability of the membrane for small charged molecules. When the electric field ceases to operate, the membrane is resealed and the cells retain the introduced molecules or ions (Chang et al., 1992; Neumann et al., 1989; Tsong, 1991; Weaver & Chizmadzhev, 1996; Zimmermann, 1986).

3.2 | Nutrient composition and glycemic index of yeast flakes

Yeast can be used for the prevention and treatment of various ailments and deficiencies, as they are a rich source of amino acids, proteins, minerals such as chromium, selenium, zinc, copper, iron, manganese, magnesium, and B vitamins (Jach & Serefko, 2018). Table 3 shows the nutritional value and glycemic index of flakes made of yeast from non-supplemented cultures and those enriched with iron and vitamin B_{12} by two different methods. Carbohydrates' content of flakes ranged from 32% to 34%. Samples C1 and C2 had higher protein content than P, whereas the highest fat content was found in the sample C1.

The samples of yeast flakes differed significantly in the ash content, which was caused by the higher iron content in the samples enriched with this element. The amount of carbohydrates, fats, and proteins influenced the caloric value of the products. Statistically significant differences were found in the caloric value of yeast flakes with the highest result for C1 sample. The glycemic index is an indispensable tool for assessing the nutritional quality of food, as a high GI of foods is associated with the increasing prevalence of diabetes,

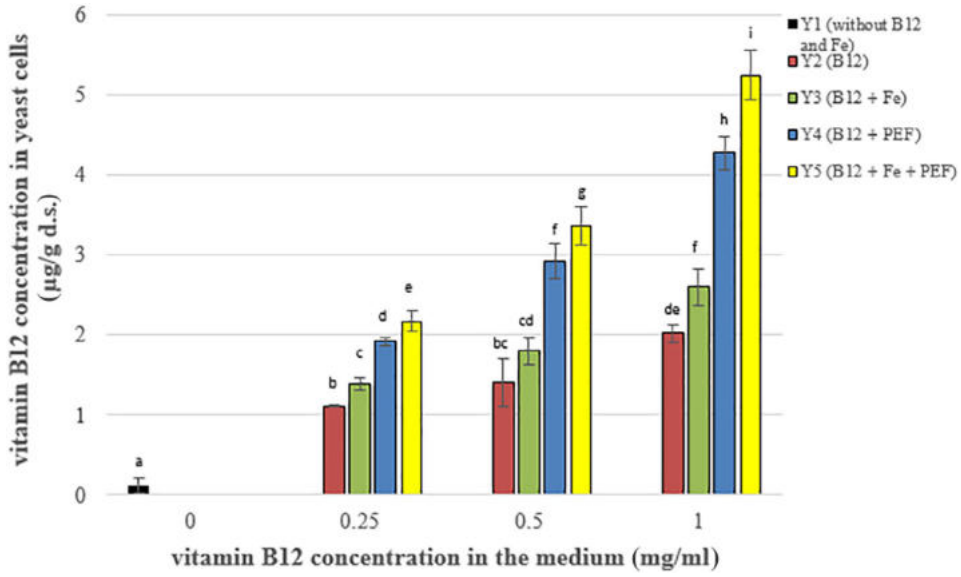

FIGURE 1 Effect of vitamin B_{12} concentration on its accumulation in yeast cells. Black bars (Y1)—control culture without iron ions and vitamin B_{12} and pulsed electric field (PEF) treatment; red bars (Y2)—cultures with vitamin B_{12} and not treated with PEF, green bars (Y3)—cultures with addition of vitamin B_{12} and iron ions (200 µg Fe^{3+}/ml medium) and not treated with PEF, blue bars (Y4)—cultures with B_{12} and treated with PEF (voltage of 1500 V, pulse width of 10 µs, treatment time 10 min, number of pulses 600, after 22 h of cultivation), yellow bars (Y5)—Cultures with vitamin B_{12} and iron ions (200 µg Fe^{3+}/ml medium) and treated with PEF (voltage of 1500 V, pulse width of 10 µs, treatment time 10 min, number of pulses 600, after 22 h of cultivation). Each value is the mean ± standard deviation ($n = 3$). Bars with the same letter (a–i) are not significantly different ($p < .05$)

TABLE 3 Composition of yeast used for the production of yeast flakes (content given on a dry mass)

Yeast	Protein (%)	Carbohydrates (%)	Fat (%)	Iron (mg/g)	Vitamin B_{12} (µg/g)
Y1	53.02 ± 0.17^{b}	33.79 ± 0.28^{b}	2.70 ± 0.14^{c}	0.13 ± 0.01^{a}	0.11 ± 0.09^{a}
Y3	52.66 ± 0.33^{b}	31.94 ± 0.12^{a}	2.50 ± 0.00^{b}	15.32 ± 0.78^{b}	2.60 ± 0.13^{b}
Y5	49.25 ± 0.10^{a}	31.99 ± 0.37^{a}	2.35 ± 0.07^{a}	38.03 ± 0.33^{c}	5.25 ± 0.31^{c}

Note: Y1—yeast without the addition of iron ions and vitamin B12 and without pulsed electric field (PEF); Y3—yeast with the addition of iron ions and vitamin B12 and without PEF; Y5—yeast with the addition of iron ions and vitamin B12 and PEF. Each value is the mean ± standard deviation ($n = 3$). Results with the same letter within a column are not significantly different ($p < .05$).

obesity and cardiovascular disease. For this reason, dietary recommendations suggest a low GI diet (<55) to prevent chronic disease (Romão et al., 2021). GI of yeast flakes was ~42, so they can complement the diet of people with insulin resistance and diabetes.

3.3 | Color of yeast flakes

Color is one of the most important sensory characteristics of food and significantly affects its overall quality as perceived by the consumers. The first impressions of food products are visual and the initial acceptance depends largely on the color of the product. Color measurement can be applied to almost any food product (Lehto et al., 2017). Table 4 shows the color of the yeast flakes surfaces in terms of L^*, a^*, b^*, and ΔE values. The higher content of iron ions and vitamin B_{12} in the sample resulted in a decrease in brightness (L^*) and yellowness (b^*). At the same time, redness (a^*) in samples enriched with vitamin B_{12} and iron increased (in proportion to the increase in iron and vitamin B_{12} content).This caused an increase in the ΔE value for yeast flakes from the sample P. The yeast enriched with iron and vitamin B_{12} (C2 and P) were clearly darker than the unenriched yeast (C1). Redness (a^*) changes may also be related to the presence of vitamin B_{12} in the samples as it is a red crystalline chemical compound (Nielsen et al., 2012). Hence the higher redness (a^*) of sample P, which contains more vitamin B_{12} than sample C2.

3.4 | The potential bioavailability of iron and vitamin B12

Animal products are a good source of vitamin B_{12} in the diet. The mean bioavailability of this vitamin in healthy humans from poultry meat ranges from 61% to 66% (Watanabe, 2007). Bioactive forms of vitamin B_{12} are not found in plant products. Some plant foods, such as seaweed and mushrooms, contain analogues of vitamin B_{12} (Watanabe & Bito, 2018). The absorption of vitamin B_{12} in the

TABLE 4 Nutritional composition, caloric value, and glycemic index (GI) of yeast flakes (content given on a dry mass) produced with yeast not enriched and enriched with iron ions and vitamin B_{12}

Yeast flakes	Proteins (%)	Carbohydrates (%)	Fat (%)	Ash (%)	Caloric value (kcal/100 g)	Caloric value (kJ/100 g)	Glycemic index (GI)
C1	53.02 ± 0.17^{b}	33.79 ± 0.28^{b}	2.70 ± 0.14^{c}	6.38 ± 0.04^{a}	387.97 ± 0.89^{c}	1623.29 ± 3.70^{c}	42.12 ± 0.11^{a}
C2	52.66 ± 0.33^{b}	31.94 ± 0.12^{a}	2.50 ± 0.00^{b}	8.81 ± 0.24^{b}	377.23 ± 0.96^{b}	1578.35 ± 4.01^{b}	42.09 ± 0.09^{a}
P	49.25 ± 0.10^{a}	31.99 ± 0.37^{a}	2.35 ± 0.07^{a}	12.58 ± 0.19^{c}	361.43 ± 0.39^{a}	1512.20 ± 1.64^{a}	41.97 ± 1.09^{a}

Note: C1—yeast flakes without the addition of iron ions and vitamin B_{12}, and without PEF; C2—yeast flakes with the addition of iron ions (200 μg Fe^{3+}/ml medium) and vitamin B_{12} (1 mg vitamin B_{12}/ml medium) and without PEF; P—yeast flakes with the addition of iron ions (200 μg Fe^{3+}/ml medium) and vitamin B_{12} (1 mg vitamin B_{12}/ml medium) and PEF. Each value is the mean ± standard deviation ($n = 3$). Results with the same letter within a column are not significantly different ($p < .05$).

TABLE 5 Values of color determinants of yeast flakes

Yeast flakes	L^*	a^*	b^*	ΔE
C1	94.65 ± 0.68^{c}	0.93 ± 0.08^{a}	11.66 ± 0.48^{c}	–
C2	91.29 ± 0.22^{b}	1.24 ± 0.04^{b}	10.86 ± 0.09^{b}	4.47
P	89.94 ± 0.93^{a}	1.36 ± 0.03^{c}	10.14 ± 0.50^{a}	6.66

Note: C1—yeast flakes without the addition of iron ions and vitamin B12 and without PEF; C2—yeast flakes with the addition of iron ions (200 μg Fe^{3+}/ml medium) and vitamin B12 (1 mg vitamin B12/ml medium) and without PEF; P—yeast flakes with the addition of iron ions (200 μg Fe^{3+}/ml medium) and vitamin B12 (1 mg vitamin B12/ml medium) and PEF. Each value is the mean ± standard deviation ($n = 3$). Results with the same letter within a column are not significantly different ($p < .05$).

terminal ileum is mediated by intrinsic factor—a glycoprotein that is produced by cells lining the stomach (Brito et al., 2018). Increased intake of gastric acid inhibitors increases the risk of vitamin B_{12} deficiency due to lack of its absorption. In contrast, calcium supplementation was found to positively influence the association between gastric acid inhibitors and vitamin B_{12} deficiency (Watson et al., 2018).

Table 5 shows the iron and vitamin B_{12} content and their potential bioavailability from the yeast flakes. The potential bioavailability of vitamin B_{12} from yeast flakes was about 3.5% in sample C2, while it was higher for sample P at about 4.3%. The higher bioavailability of vitamin B_{12} from sample P may be due to its higher content in this sample than in C2. However, the bioavailability of this vitamin from the tested samples was still low. One way to increase it could be to add vitamin C to the yeast flakes. The absorption of vitamin B_{12} also depends on its dose in the product. It is likely that the intestinal absorption of vitamin B_{12} via intrinsic factors is a maximum of about 1.5–2.0 μg per meal under physiological conditions. Therefore, the bioavailability of vitamin B_{12} may drop significantly with increasing intake of this vitamin in the meal (Watanabe & Bito, 2018). We observed that yeast flakes contained 82.5% less vitamin B_{12} than fresh yeast (5.25 μg/g dry weight of yeast and 0.92 μg/g dry weight of yeast flakes). The difference in vitamin B_{12} content between these samples is likely due to the high temperature of the yeast flake preparation process (Bajaj & Singhal, 2021). In a pilot study with five participants performed by Garrod et al. (2019), it was determined whether enriching flour with vitamin B_{12} yielded a bread product with intact vitamin B_{12} content. This study showed that when vitamin B_{12} is added to flour, it survives fermentation processes and high temperatures during baking and retains approximately 50% bioavailability when given to healthy individuals in low doses. The recommended intake of vitamin B_{12} for adults is 2.4 μg/day. This recommendation assumes that the usual bioavailability of low doses of the vitamin in crystalline form is 60%, whereas for the same amount in foods such as meat and fish it is 50% (Wolffenbuttel et al., 2019).

The yeast flakes C1 and C2 contained, respectively, ~12 mg and ~1420 mg iron in 100 g dry mass. Using yeast enriched with iron under PEF conditions to prepare yeast flakes increased the iron content of the flakes to nearly 3600 mg iron/100 g dry mass. Such high amounts of iron in yeast flakes are due to the use of yeast alone in their production (Table 6).

The potential bioavailability of iron from yeast flakes is about 6.8% for sample C2 and about 10% for sample P (Table 6). No nutrient has as many dietary factors that affect its bioavailability. Factors that inhibit iron utilization result in nearly 80% of all iron from the diet ending up in the feces (Banjari & Hjartåker, 2018). Iron absorption is enhanced by vitamin C (can increase dietary iron absorption by up to 10%) while calcium has the opposite effect (Watson et al., 2018). For this reason, as with vitamin B_{12}, vitamin C could be added to the product to increase the bioavailability of iron.

Numerous studies have been conducted on the bioavailability of iron from iron-fortified products, but comparing results can be problematic due to differences in study methodology (Diego Quintaes et al., 2017). In the study by Pizarro et al. (2006) bread enriched with ferrous sulfate was prepared. The content of iron in bread was 0.47 mg in 100 g. The potential bioavailability of iron from this product was 10.5%, but the authors used an in vivo method so it is difficult to compare it with our results. In a study by Doumani et al. (2020) the total iron content in humus samples was on average 4.7 mg/100 mg. In this case, the in vitro bioavailability of iron from garlic enriched humus was 13.6%, while from this product not containing garlic it was

8.2%. In the study by Bryszewska et al. (2019) the in vitro iron bioavailability was determined using the Caco-2 cell line. The bioavailability of iron after digesting iron-enriched bread ranged from 41.45% to 99.31%. Wahengbam et al. (2019) investigated iron bioavailability from rice fortified with iron and folic acid. The potential bioavailability of iron in the enriched unground rice was 57.6% so it was higher than that of our product. This was most likely influenced by other compounds, such as in the study by Wahengbam et al. (2019) it was folic acid, which increases iron absorption (Jyrwa et al., 2020), and in the case of the study by Doumani et al. (2020), garlic and vitamin C-containing raw materials were used to make hummus, which can also increase iron absorption (Nahdi et al., 2010; Subroto et al., 2021).

3.5 | Antioxidant activity

The antioxidant activity of food products depends on the presence and content of bioactive compounds. Consumption of bioactive compounds reduces the risk of non-communicable diseases (Granato et al., 2018). The antioxidant activity of yeast flakes was investigated through the ability of the extracts to inhibit DPPH$^{\bullet}$ and ABTS$^{\bullet+}$. For antioxidant activity against both free radicals, no significant differences ($p < .05$) between samples were found. The highest values of antioxidant activity against ABTS$^{\bullet+}$ were determined for yeast flakes C1, and the lowest for yeast flakes P (0.8 and 0.77 mMTE, respectively). For DPPH$^{\bullet}$ the relationship was the same, C1 yeast flakes had the highest antioxidant activity and P yeast flakes the lowest (1.93 and 1.81 mMTE, respectively). Increasing the vitamin B_{12} content by application of PEF did not affect the antioxidant activity of trial P. The lower antioxidant capacity of products with higher vitamin B_{12} and iron contents may be due to the nature of iron, as it is a metal with redox activity that is involved in electron transfer reactions, which in turn leads to the production of oxidants capable of oxidizing cellular components (Zago & Oteiza, 2001). In turn, the presence of vitamin B_{12} improves antioxidant properties, which may compensate for the effect of iron on antiradical activity. Lower vitamin B_{12} levels are associated with increased prooxidant levels and decreased antioxidant status (Van De Lagemaat et al., 2019). Supplementation with

TABLE 6 Iron and vitamin B_{12} content in 100 g of dry mass and potential bioavailability of iron from yeast flakes

Yeast flakes	Iron content (mg/100 g)	The potential bioavailability of iron (%)	Vitamin B_{12} content (μg/100 g)	The potential bioavailability of vitamin B_{12} (%)
C1	11.98 ± 1.01^{a}	5.16 ± 0.60^{a}	1.03 ± 0.29^{a}	0.00^{a}
C2	1424.42 ± 0.78^{b}	6.77 ± 0.36^{b}	64.90 ± 3.18^{b}	3.53 ± 0.76^{b}
P	3593.10 ± 4.33^{c}	10.13 ± 0.08^{c}	92.42 ± 3.91^{c}	4.31 ± 0.44^{c}

Note: C1—yeast flakes without the addition of iron ions and vitamin B_{12} and without pulsed electric field (PEF); C2—yeast flakes with the addition of iron ions (200 μg Fe^{3+}/ml medium) and vitamin B12 (1 mg vitamin B_{12}/ml medium) and without PEF; P—yeast flakes with the addition of iron ions (200 μg Fe^{3+}/ml medium) and vitamin B_{12} (1 mg vitamin B_{12}/ml medium) and PEF. Each value is the mean ± standard deviation ($n = 3$). Results with the same letter within a column are not significantly different ($p < .05$).

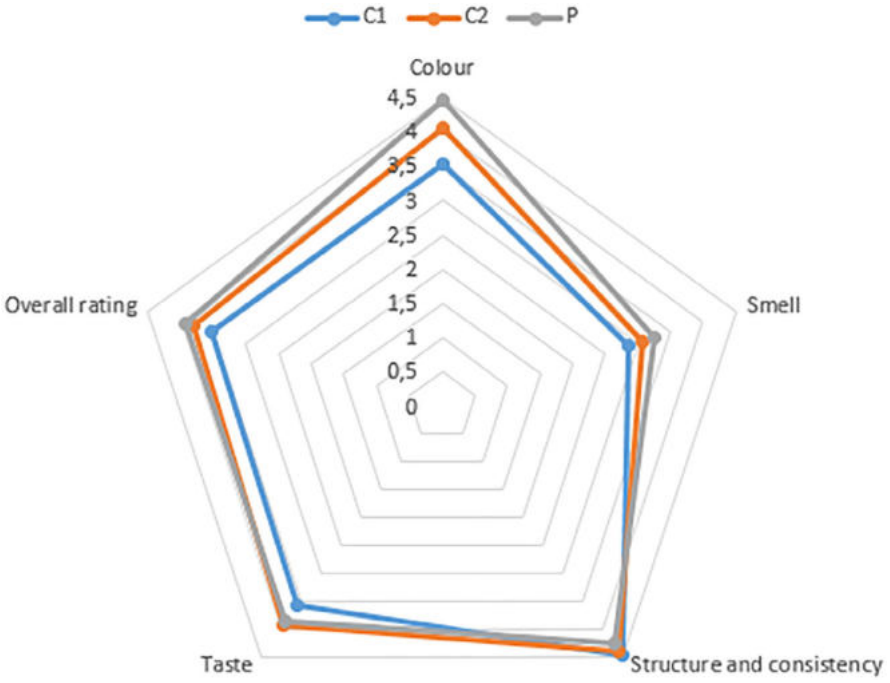

FIGURE 2 The 5-point rating radar chart. C1—Yeast flakes without the addition of iron ions and vitamin B_{12} without pulsed electric field (PEF); C2—Yeast flakes with the addition of iron ions (200 μg Fe^{3+}/ml medium) and vitamin B_{12} (1 mg vitamin B_{12}/ml medium) without PEF; P—Yeast flakes with the addition of iron ions (200 μg Fe^{3+}/ml medium) and vitamin B_{12} (1 mg vitamin B_{12}/ml medium) and PEF. Each value is the mean ± standard deviation ($n = 3$)

physiologically relevant concentrations of cyanocobalamin (a form of B_{12} commonly used in supplements) has been shown to reduce superoxide levels in the cytosol and mitochondria (Kostyuk et al., 2013). In addition, enzymatically processed vitamin B_{12} acts as a direct scavenger of superoxides (Chan et al., 2018) and may stimulate free radical scavenging by preserving glutathione (Karamshetty et al., 2016).

3.6 | Sensory evaluation

The acceptability of a food product by the consumer depends on many factors. The sensory and health-promoting properties of the product are of the greatest importance whether a product will be accepted or rejected (Mir et al., 2014). The high nutritional value of the food product makes it more acceptable to consumers. For this reason, the use of mineral-enriched yeast for the production of functional food or as a supplement is becoming more and more popular (Nowosad & Sujka, 2021; Pas et al., 2007; Pirman & Orešnik, 2012; Wang et al., 2011). However, the metallic taste and discoloration of iron-fortified products are often cited as the reasons for unsuccessful attempts to bring such products to the market and gain consumer acceptance (Bovell-Benjamin & Guinard, 2003).

Iron compounds that are poorly soluble in gastric juice do not affect the sensory properties of the product in which they occur (Hurrell, 1997), while these compounds which has very good solubility can cause changes in the color and taste of food products due to their participation in the fat oxidation process (Eichler et al., 2019).

The results of overall rating for yeast flakes P and C2 were the highest and they did not differ significantly (Figure 2). The yeast flakes C1 were rated the lowest. Color was rated higher for the product P than C1 and C2 (no statistically significant differences were found between these two samples, $p < .05$). Statistically significant differences were found between samples P, C2, and C1. These differences were due to dark red color of flakes C2 and P caused by the presence of vitamin B_{12}. There were no significant differences between the yeast flakes in such features as: structure, consistency, and taste. The quality of yeast flakes C1 was rated as satisfactory, while in the case of C2 and P yeast flakes it was good. The presence of iron did not change the sensory characteristics of the products.

4 | CONCLUSIONS

In our study, we enhanced accumulation of iron and vitamin B_{12} in yeast cells using pulsed electric field. The application of PEF significantly increased (by 50%) the concentration of vitamin B_{12} in yeast compared to that supplemented with this vitamin but without PEF treatment. Yeast enriched with iron and vitamin B_{12} was used to produce yeast flakes. The obtained yeast flakes contained 3.6 g of iron and 92.4 μg of vitamin B_{12} per 100 g of product. The potential bioavailability of iron was 10% and of vitamin B_{12} 4.31%. The yeast flakes were characterized by a low glycemic index. The nutritional composition of yeast flakes shows that this product can be a very good source of iron and vitamin B_{12} for people exposed to a deficiency of these ingredients, for example, during the use of plant diets. Due to their low glycemic index, yeast flakes can also be used by diabetics. In the future, the composition of the flakes should be modified in order to increase the bioavailability of both components, as well as the bioavailability should be tested by in-vivo methods. It would also be desirable to make attempts to increase the scale of production of yeast flakes with a view to their possible introduction to the market.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This study did not involve any animal or human testing.

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