

Novel Qualitative Screening Approach for Determination of ABE in Fermentation Products

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Some of genus *Clostridium* bacteria are natural producers of biobutanol via acetone-butanol-ethanol fermentation. Butanol is recognized as an alternative fuel for many years. Unfortunately, biobutanol production is tedious and requires constant process control and monitoring due to strict requirements with respect to sterility, anaerobic atmosphere, and other factors. Furthermore, butanol is a well-known ABE fermentation inhibitor and its accumulation in the fermentation broth often results in cell death. Conventionally, gas chromatography or high-performance liquid chromatography is used for analysis of butanol in fermentation products. Even though the methods are highly efficient, they are tedious, expensive, and time-consuming. Within this study we designed a rapid and low-cost qualitative screening approach to determine metabolic product formation in ABE fermentation within 10 minutes using spectrophotometry and salting-out. The method allowed to determine butanol at concentrations > 3 % in samples containing fermentation broth and other solvents. The results demonstrated that after the set-up of test conditions, spectrophotometric ABE detection can be used as rapid screening tool in fermentation studies and butanol production monitoring.

1. Introduction

Butanol fermentation is a natural process for some bacteria first discovered by Louis Pasteur in 1861. In 1912 *Clostridium acetobutylicum* was first described as being able to convert starch to acetone, butanol and ethanol (Ezeji et al., 2004). The process is called acetone-butanol-ethanol (ABE) fermentation and typical mass proportion for produced solvents is 3:6:1 in the solventogenic fermentation phase (Xue, Cheng, 2019). Initially ABE fermentation was used in industrial butanol production plants (Lee et al., 2008). However, from mid-20th century it was replaced with petrochemical process (Moon et al., 2016). Traditional ABE fermentation was declared ineffective due to high substrate costs, low butanol yield (<3%) and high cost of product recovery (Abo et al., 2019; Qureshi et al., 2006). To overcome these hurdles, understanding that butanol is a superior liquid fuel to ethanol (higher energy content, lower volatility, no corrosivity) and has high potential to replace fossil fuels, multiple strategies have been developed within the last decades for more efficient biobutanol production via ABE fermentation (Stoller et al., 2019). These include the use of renewable resources as feedstock, introduction of genetically modified microorganisms able to produce more butanol under simplified fermentation conditions, continuous fermentation processes and “in situ” product recovery techniques (Abo et al., 2019).

Butanol itself is a natural inhibitor of ABE fermentation process, and, thus, butanol concentration should be kept low (8 – 10 g/L) in the fermentation broth (Yen et al., 2011). For various *Clostridia* full inhibition of fermentation was observed at butanol concentrations around 6 g/L (Birgen et al., 2018) Due to the extremely high costs of distillation (Schack et al., 2020), alternative recovery techniques, like, gas stripping, liquid-liquid extraction, membrane separation, pervaporation (Kiss et al., 2019) and salting-out (Wen et al., 2018) have been suggested for butanol recovery. Despite the high potential of salting-out, it is not widely recognized on an industrial scale. Requirement for large quantities of salt and inability to recover it are the main reasons. At the same time, use of the salting-out is widely practiced as pre-treatment step in gas chromatography assays (Fu et al., 2020) to assess butanol yields in the industrial production and research studies. Being highly precise, these methods are expensive, labour, and time-consuming. Often the challenge of chromatographic methods

lay in the reduction of time for sample analysis without affecting the precision of the method (Picó, 2020) and ability to determine the target substance at various stages of production and changing substrates, e.g., fermentation of starch or hydrolysates of various lignocellulosic biomass (Birgen et al., 2019). A new approach for large scale ABE fermentation process monitoring is flow cytometry (Potakova et al., 2013). This method is based on visual cell differences in acidogenic and solventogenic phase, thus, cannot be used for direct product assessment. Maiti et al. (2015) have described a method for combination of butanol salting-out and spectrophotometric measurement using diquat. Despite the fascinating results, the research involved measurements of relatively high butanol concentrations, typically not observed in fermentation of natural butanol producing *Clostridia*. Furthermore, the impact of ethanol, acetone and growth medium were not addressed adequately. The aim of this study was to re-construct and validate the spectrophotometric butanol measurement assay with salting-out and diquat for rapid and cost-effective monitoring of butanol in fermentation studies. Method adjustment included estimation of butanol detection limits, analysis of artificial and natural fermentation samples and evaluation of impact from other fermentation products, e.g., ethanol and acetone.

2. Materials and Methods

2.1 Reagents for spectrometric quantification

1 N NaOH solution was made beforehand and stored at +4 °C. Diquat solution containing 1 g/L diquat-dibromide-mono-hydrate (SigmaAldrich, Germany) was prepared by dissolving diquat-dibromide-mono-hydrate in 10 mL of 0.1 M acetic acid buffer solution (pH=4), distributed in 2 mL microtubes and stored at +4 °C until use. To prepare the test reagent, 20.4 µL of diquat solution and 20.4 µL of 2-mercapto ethanol (SigmaAldrich, Germany) was added to 2959.2 µL of 1N NaOH in a test tube. The solution was vortexed for 5 s and used within 5 minutes after preparation or when color change from yellowish to red was observed.

2.2 Screening of different salts for salting-out

To determine the most efficient salt for salting-out butanol, 92.3 g/100 mL K_3HPO_4 ; 150 g/100 mL K_2HPO_4 ; 22.6 g/100 mL KH_2PO_4 ; 35.9 g/100 mL NaCl; 75.4 g/100 mL $(NH_4)_2SO_4$; 21.5 g/100 mL Na_2CO_3 were tested. The concentrations were selected based on the solubility of each salt. Each sample was tested by measuring 10 % and 30 % 1-butanol (ACROS Organics) solution in sterile distilled water according to method described in subsection 2.3.

2.3 Butanol spectrophotometric quantification

To spectrophotometrically determine butanol concentration, a modified protocol described by Maiti et al. (2015) was used. In brief, selected salt (section 3.1.) was mixed with 3 mL of test solution (for standard curve set-up 0.1, 0.25, 0.5, 1, 2, 3, 4, 5, 10 % of butanol in distilled water) and 3 mL of salting-out reagent solution. To increase the detection limit of the method, 1 mL of pure butanol was added. The obtained solution was vortexed for 30 s and incubated for 2 min. Within this time formation of 2 distinct layers was observed. The upper layer should turn purple (Figure 1). Then 1 mL of it was removed and absorption at 520 nm (Genesys 150, ThermoScientific, USA) was measured. To obtain standard curve, absorption was plotted against the used concentration. All measurements and tests were prepared in at least triplicates.

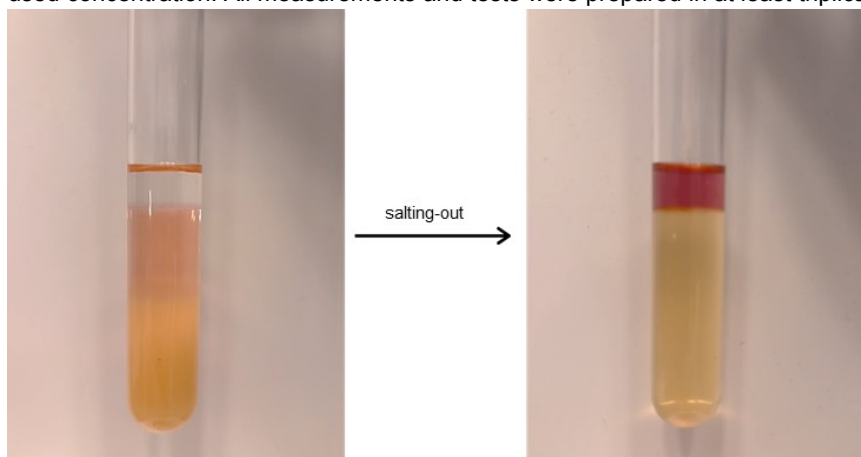


Figure 1: Sample before and after salting-out reaction with K_3PO_4 and 10% butanol

2.4 Simulated ABE fermentation product test

Further the ABE product salting-out method was customized for use in fermentation samples. This included assessment of the following factors: presence of culture media, acetone, and ethanol. First, distilled water was replaced with *Clostridium* spp. growth medium (5 g/L peptone, 5 g/L Lab-Lemco, 10 g/L yeast extract, 0.5 g/L KH_2PO_4 , 0.5 g/L $\text{K}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 0.2 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g/L $\text{FeSO}_4 \cdot 2\text{H}_2\text{O}$, 0.01 g/L NaCl, 0.001 g/L p-aminobenzoic acid; 0.00001 g/L biotin; 30 g/L dextrose) as diluent in test solutions. Before tests, growth medium was sterilized in 121 °C for 15 min and filtered through 0.10 µm membrane filter to ensure a clear solution. 0, 0.1, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 12.0, 15.0 % butanol solutions in growth media were used for standard curve set up. All measurements were performed three to five times.

Further separate absorption curves for three main ABE fermentation products were plotted using 1-butanol, ethanol and acetone solutions (0.1 to 15 %). Then, a simulated ABE fermentation product mix was prepared at the ratio of 3:6:1 (acetone:butanol:ethanol). To determine the effect of acetone and ethanol on butanol spectrophotometric measurements, all concentrations used in the standard curve construction were assayed.

2.5 ABE fermentation studies

To evaluate the adjusted method for fermentation samples, ABE fermentation studies in laboratory scale glass bioreactor with working volume of 2-4.5 L and one speed-controlled standard Rushton turbine type agitator with 6 blades (Biotechnical Centre, Riga, Latvia) were performed. Stock culture of *Clostridium beijerinckii* DSM 6422 maintained under anaerobic conditions (37 °C) was inoculated in growth medium (section 2.4.) for 12 h before fermentation studies and incubated under anaerobic conditions (Anaerojar, Oxoid Ltd, UK). Then the culture was centrifuged for 2 minutes at 3387 rcf (Ohaus, Poland), supernatant discarded and replaced with fresh growth medium. Further the culture was aseptically poured into pre-sterilized bioreactor filled with 3 L of sterile growth medium and incubated at 37 °C with stirring regime of 50 rpm. To ensure anaerobic conditions, inert nitrogen gas was supplied to the system for 5 min through 0.10 µm membrane filter. Samples were collected after 0, 12, 24, 36, 48, 54 and 120 h. Each time 30 mL of sample was collected and filtered through 0.10 µm membrane filter, divided into 10 mL portions for salting out. Samples were stored at -18 °C until use.

3. Results and discussion

3.1 Screening of salts for salting-out

To efficiently employ spectrophotometric butanol measurements with diquat, most appropriate salt for salting-out process must be selected. Numerous studies have been performed to assess chemical equilibrium and optimal properties of various salts and have demonstrated the appropriateness of one and inferiority of others (Fu et al., 2020; Maiti et al., 2015; Wen et al., 2018). Some of the recommended salts are K_2HPO_4 , $\text{K}_4\text{P}_2\text{O}_7$, K_3PO_4 . To evaluate the efficiency and suitability of the method for ABE fermentation samples, initial salt screening was performed with 6 most common salts (Figure 2). Salt screening results with 10% butanol solution presented two potentially efficient salts for salting-out of butanol from fermentation samples – K_3PO_4 and Na_2CO_3 . These were soluble without any need for additional heating or stirring and produced butanol absorption values >1.0 when compared to other salts.

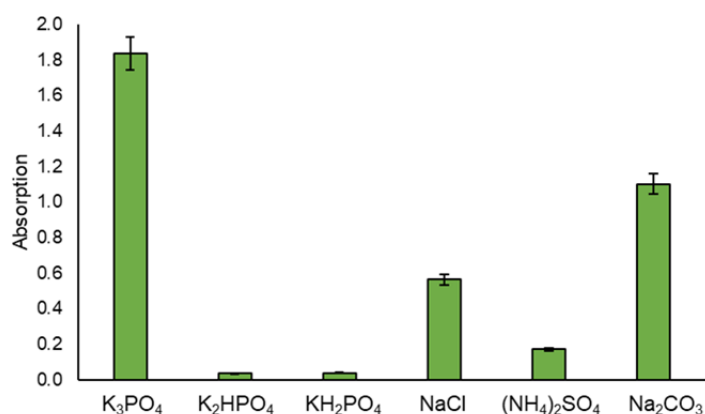


Figure 2: Absorption values of different salts for salt screening tests with 10 % butanol. Standard deviation represents the average value of 3 individual measurements

From both salts, K_3PO_4 was preferred due to its high solubility at room temperature, prior recognizability (Maiti et al., 2015), efficiency also at 30 % butanol concentrations (data not shown) and compatibility of salt quantity needed for one test with standard glass test tubes, that is essential in rapid laboratory assay development (reduced amount of laboratory materials for testing).

3.2 Butanol standard curve set-up

During salting-out experiments employing > 10% butanol samples, it was estimated that, time and working temperature is extremely affecting the salting-out reaction. The reduced diquat-dibromide-mono-hydrate in alkaline solutions is stable only for 5 minutes (WHO & FAO, 2014). Sample preparation for measurements takes up to 4 minutes. This limits the time frame available for measurements and as the reagent decays, measurements should be done at the exact same time for each sample. Furthermore, reagent and sample temperature had a significant effect on sample absorption. At +4 °C the absorption value for 10% pure butanol solution was 0.633, while at room temperature the absorption value for the same sample was 0.776. The difference in absorption values suggests on the need to set up a stringent protocol for sample measurements. To achieve more representative sample conditions, the distilled water was replaced with fermentation broth in preparation of the butanol standards. When these samples were salted-out, layer of brown particles formed between the purple butanol and yellowish growth medium layer. This was further considered in all ABE samples and subsequently all samples were filtered prior analysis. Butanol absorption values for 0.1, 0.25, 0.5, 1 and 2 % solutions varied drastically each time and no mathematical relation for describing this fluctuation could be calculated. After the repetitive measurements a detection limit for butanol was set at 3 %. Since during ABE fermentation other solvents are produced, effect of ethanol and acetone was estimated with the selected protocol. No absorption measurements for ethanol were obtained since none of the ethanol samples formed two separate layers as seen in Figure 1 within 2 minutes of incubation. The solution became unclear after mixing and layer formation occurred only after 15 minutes. As recognized (Maiti et al., 2015), diquat reagent degrades over time and after 15 minutes absorption measurements are not objective anymore. Samples of acetone solution formed two separate layers and the upper layer turned purple after 2 minutes. At the same time no correlation between acetone concentration and absorption was observed (Figure 3). The absorption values for acetone remained relatively constant – ranging from 0.291 to 0.391. At the same time, butanol absorption units ranged from 0.906 ± 0.104 for 3 % butanol to 0.707 ± 0.014 for 15 % butanol in growth media solvent. Thus, it was estimated that acetone has no or only minor impact on salting-out butanol from ABE samples and its subsequent spectrometric measurement.

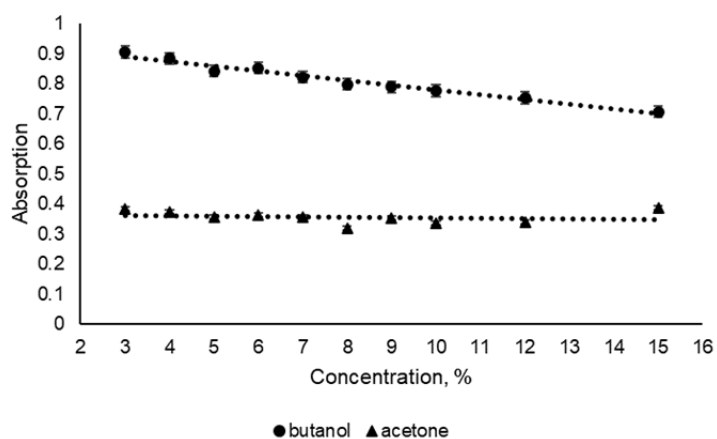


Figure 3: Absorption values for various concentrations of butanol and acetone in individual standard solutions when growth media is used as solvent instead of water. Standard deviation represents average value from at least 3 individual measurements

The obtained butanol absorption curve (Figure 3) was further used to calculate a mathematical relation between butanol concentration (3 % – 15 %) and absorption at 520 nm for pure butanol solutions, where y is the absorption and x is butanol concentration (Eq 1).

$$y = -0.016x + 0.940 \quad (1)$$

3.3 Butanol measurements in simulated and fermented ABE samples

Simulated ABE fermentation product tests were performed with acetone:butanol:ethanol mixes in the range 3:6:1 (keeping the butanol from 3 to 15 %) and followed the same trend (Eq. 1) as calculated with pure butanol of various concentrations; however, significant ($p < 0.05$) reduction in absorption values was obtained. Absorbance for 3 % butanol solution was 0.618 ± 0.013 instead of 0.906 ± 0.104 as determined in pure butanol samples (Figure 3). In general, an average of 33.0 ± 2.7 % decrease in all absorption values was observed for all samples. As before, the detection limit for butanol in ABE samples was found to be 3 % butanol. Due to the relatively high detection limit, the method cannot be used for analytical measurements or sensitive process development, at the same time by performing certain adjustments in the protocol, the obtained detection limit was significantly lower than previously reported (Maiti et al., 2015). As a result, a standard curve was constructed for butanol concentration values from 3 % to 15 % ($R^2=0.993$) in solutions containing growth media, acetone and ethanol (Figure 4). Mathematical relation was computed for the curve where y is the absorption at 520 nm and x is the respective butanol concentration (Eq. 2). Absorption values for chosen concentrations ranged from 0.618 (3 %) to 0.438 (15 %) and values exceeding maximum absorption should be considered of concentration lower than 3 %.

$$y = -0.016x + 0.671 \quad (2)$$

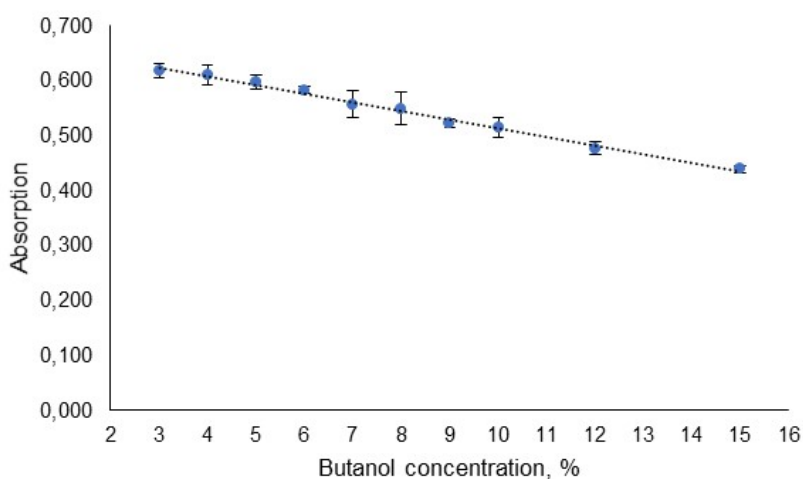


Figure 4: Standard curve for butanol detection in ABE fermentation samples. Standard deviation represents the average value from at least 3 individual measurements

Within this study adjustments in spectrophotometric butanol quantification with diquat was performed to assess the impact of other solvents, fermentation media and analysis conditions. Multiple factors appeared crucial for efficient application of this technique. At the same time after thorough set-up of the protocol, rapid tool for analysis of ABE products was generated. As reported by Terracciano and Kashket (1986) solventogenesis in ABE fermentation with *Clostridium* spp begins after around 24 h. Salting-out method could be applied for samples after 24 h, as confirmed by real ABE fermentation broth sample tests. Absorption for 0 h, 12 h and 24 h samples was 0.727, 0.845 and 0.898 respectively, which exceed maximum absorption – 0.618 for 3 % butanol in ABE samples. Absorption of 36 h and 48 h samples varied depending on the experimental run remained lower than 0.618 suggesting this method as potential tool for fermentation process monitoring after 24 h. This method is more efficient compared to other ABE fermentation process screening methods. Tests can be performed in less than 10 minutes and this method is less expensive compared to chromatography or cytometry. Moreover salting-out method allows to analyze samples directly from fermentation reactors after filtration. Nevertheless, there are certain limitations to this screening method regarding available time and changes in temperature and need to construct standard curve for each specific system, since the results strongly depend on absolute absorption values of the system. Furthermore, the measurement conditions should remain constant because of temperature and strict incubation time.

4. Conclusions

Rapid, effective, and low-cost screening method for detection of ABE fermentation products in fermentation broth was designed using diquat reagent and K_3PO_4 in salting-out reaction employing a single protocol for

both construction of standard curve and sample analysis. Spectrophotometric detection can be used to detect butanol at concentrations as low as 3 % within 10 minutes, at the same time prior introduction of the approach stringent protocol and construction of relevant standard curve must be performed to validate the detection limits and influence of acetone.

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References

- Abo, B. O., Gao, M., Wang, Y., Wu, C., Wang, Q., & Ma, H., 2019, Production of butanol from biomass: recent advances and future prospects, *Environmental Science and Pollution Research*, 26(20), 20164–20182.
- Birgen, C., Dürre, P., Preisig, H.A., & Wentzel, A., 2019, Butanol production from lignocellulosic biomass: revisiting fermentation performance indicators with exploratory data analysis, *Biotechnology for Biofuels* 12, 167, 1–15.
- Birgen, C., Markussen, S., Wentzel, A., Preisig, H.A., 2018, The Effect of Feeding Strategy on Butanol Production by *Clostridium beijerinckii* NCIMB 8052 using Glucose and Xylose, *Chemical Engineering Transactions*, 65, 283-288.
- Ezeji, T. C., Qureshi, N., & Blaschek, H. P., 2004, Butanol fermentation research: upstream and downstream manipulations, *Chemical Record*, 4(5), 305–314.
- Fu, C., Li, Z., Song, W., Yi, C. & Xie, S., 2020, A new process for separating biofuel based on the salt + 1-butanol + water system, *Fuel*, 278, 118402, 1–8.
- Kiss, A.A., Pătrașcu, I., Bîldea, C. S., 2019, From substrate to biofuel in the acetone-butanol-ethanol process, Chapter in: Basile, A., Dalena, F. (Eds.) *Second and Third Generation of Feedstocks: The Evolution of Biofuels*, Elsevier, Amsterdam, The Netherlands, pp. 59–82.
- Lee, S. Y., Park, J. H., Jang, S. H., Nielsen, L. K., Kim, J., & Jung, K. S., 2008, Fermentative butanol production by clostridia, *Biotechnology and Bioengineering*, 101(2), 209–228.
- Maiti, S., Sarma, S. J., Brar, S. K., Bihan, Y. Le, Drogui, P., Buelna, G., Verma, M., & Soccol, C. R., 2015, Novel spectrophotometric method for detection and estimation of butanol in acetone-butanol-ethanol fermenter, *Talanta*, 141, 116–121.
- Moon, H. G., Jang, Y.-S., Cho, C., Lee, J., Binkley, R., & Lee, S. Y., 2016, One hundred years of clostridial butanol fermentation, *FEMS Microbiology Letters*, 363(3).
- Patakova, P., Linhova, M., Rychtera, M., Paulova, L., & Melzoch, K., 2013, Novel and neglected issues of acetone-butanol-ethanol (ABE) fermentation by clostridia: *Clostridium* metabolic diversity, tools for process mapping and continuous fermentation systems, *Biotechnology Advances*, 31(1), 58–67.
- Picó, Y., 2020, Chromatography–mass spectrometry: Recent evolution and current trends in environmental science, *Current Opinion in Environmental Science and Health*, 18, 47–53.
- Qureshi, N., Li, X.L., Hughes, S., Saha, B.C., Cotta, M.A., 2006, Butanol production from corn fiber xylan using *Clostridium acetobutylicum*, *Biotechnology Progress*, 22(3), 673–680.
- Schack, D., Jastram, A., Liesche, G., & Sundmacher, K., 2020, Energy-Efficient Distillation Processes by Additional Heat Transfer Derived From the FluxMax Approach, *Frontiers in Energy Research*, 8, 134.
- Stoller M., Bravi M., Russo P., 2019, Development of a Process for N-butanol Recovery from Abe Wastewater Streams by Membrane Technology, *Chemical Engineering Transactions*, 74, 937-942.
- Terracciano, J. S., & Kashket, E. R., 1986, Intracellular Conditions Required for Initiation of Solvent Production by *Clostridium acetobutylicum*, *Applied and Environmental Microbiology*, 52(1), 86–91.
- Wen, H., Chen, H., Cai, D., Gong, P., Zhang, T., Wu, Z., Gao, H., Li, Z., Qin, P. & Tan, T., 2018, Integrated in situ gas stripping–salting-out process for high-titer acetone–butanol–ethanol production from sweet sorghum bagasse, *Biotechnology for Biofuels*, 11, 134, 1–12.
- World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO), 2014, Pesticide residues in food 2013: evaluations 2013, part I - residues, *FAO Plant Production and Protection Paper No. 220*, Rome, Italy.
- Xue, C., & Cheng, C., 2019, Butanol production by *Clostridium*, Chapter in: Ge X., Li Y. (Eds.), *Advances in bioenergy*, Vol. 4, Elsevier, 35–77.
- Yen, H.-W., Li, R.-J., & Ma, T.-W., 2011, The development process for a continuous acetone–butanol–ethanol (ABE) fermentation by immobilized *Clostridium acetobutylicum*, *Journal of the Taiwan Institute of Chemical Engineers*, 42(6), 902–907.