# Bioprocess development for the production of succinic acid from orange peel waste

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**Abstract**

A preliminary study has been conducted for the development of a bioprocess targeting the valorization of orange peel waste (OPW). Essential oils and pectin were recovered from OPW through distillation, acid hydrolysis and precipitation with ethanol. Optimal conditions for dilute-acid hydrolysis were investigated via estimation of the sugars released and fermentation. Hydrolysis conditions of 109 $^o$C for 20 min and 116 $^o$C for 10 min using 5 % of dry raw material produced the highest sugar yields at 0.76 and 0.77 ($g_{total\ sugars}\ g_{dry\ raw\ material}^{-1}$) respectively. In order to test the efficiency of enzyme hydrolysis as a pretreatment method for OPW valorization, cellulase production from *T. reesei* was investigated, which was maximized following 5 days of cultivation. Furthermore, elemental analysis in hydrolyzates from dilute-acid hydrolysis and a combination of acid and enzyme hydrolysis was performed. The results indicate that during acid/enzyme hydrolysis, high concentrations of $Mg^{2+}$ and $Ca^{2+}$ ions are liberated in contrast to dilute-acid hydrolysis. *A. succinogenes* fermentations of glucose and fructose were performed and the succinic acid yields achieved were 0.66 ($g_{succinic\ acid}\ g_{glucose}^{-1}$) and 0.33 ($g_{succinic\ acid}\ g_{fructose}^{-1}$) respectively, while galactose was not fermented. Overall, OPW may serve as a promising raw material for simultaneous production of essential oils, pectin and succinic acid.

**Keywords**

orange peel waste, industrial biotechnology, fermentation, succinic acid, *A. succinogenes*, *T. reesei*

## 1. Introduction

The global citrus production constitutes over $88 \times 10^6$ tons annually, while industrial OPW generation reaches approximately $15 \times 10^6$ tons [1]. During processing of the fruit, half of its mass is extracted as juice, while the rest remains as peel waste consisting of peels, seeds and segment membranes [1, 2]. Citrus fruits of industrial interest are oranges, lemons, grapefruits and mandarins, with oranges being the most important consisting 82 % of the total production according to Marin *et al* [1]. Current management practices for OPW include its use as animal feed or disposal in landfills. However, the composition of the peel renders OPW a promising feedstock for the biotechnological production of added-value products through the biorefinery platform. Specifically, OPW is rich in pectin and essential oils, while the high cellulose, hemicellulose and soluble sugars content can be valorized [3].

Components of OPW such as pectin, cellulose and hemicellulose must be pretreated prior to fermentation. Previous studies evaluated commercial enzymes such as pectinase, cellulase and β-glucosidase for breaking of pectin, cellulose, hemicellulose and β-glycosidic bonds [4]. Pourbafrani *et al* [5] studied dilute-acid hydrolysis of polysaccharides contained in OPW at high temperatures, whereas previous studies employed microorganisms for enzyme production, which was then consequently used for enzyme hydrolysis of lignocellulosic biomass. Specifically, *Trichoderma reesei* and *Aspergilus niger* are considered as the most suitable microorganisms for the preparation of cellulase and β-glucosidase, respectively [6, 7].

Soluble sugars and monosaccharides liberated through hydrolysis could serve as a nutrient rich fermentation broth for the production of succinic acid, which is predicted to be one of the most important biobased platform chemicals used for the manufacture of various added-value products [8]. Succinic acid ($C_4H_6O_4$) is a di-carboxylic acid produced mainly by chemical technologies such as catalytic hydrogenation, paraffin oxidation and electrolytic reduction of maleic acid or anhydride [9]. The biobased production of succinic acid has a series of advantages compared to its chemical production due to high theoretical yield and environmental friendly impact.

Various microorganisms such as *Mannheimia succiniciproducens*, *Anaerobiospirillum succiniciproducens*, *Basfia succiniciproducens* and *Actinobacillus succinogenes* have been tested for succinic acid production in previous studies [9, 10]. Among the strains examined, *A. succinogenes* is predicted to be one of the most promising industrial succinic acid-producing microorganism because it has the ability to utilize $CO_2$ and to produce high concentrations of succinic acid. *A. succinogenes*, isolated from bovine rumen, is a capnophilic and mesophilic bacterium which is capable of valorizing monosaccharides under anaerobic conditions [10].

The aim of the current work was to conduct a preliminary study for the development of an OPW bio-refinery (Fig. 1). Specifically, the bioprocess presented in this work targets, following the removal of essential oils from OPW, optimization of dilute-acid hydrolysis conditions, pectin recovery as well as investigation of optimal cultivation time for cellulolytic enzyme production by *T. reesei*. The release of metal ions during hydrolysis has been evaluated, while succinic acid fermentations were conducted using simple sugars and OPW hydrolyzates.

## 2. Methods

### 2.1. Pretreatment of OPW and vacuum distillation

OPW was obtained from a local juice factory (KEAN, Limassol, Cyprus) and stored at -20 °C until further use. The first step of OPW pretreatment required extraction and collection of essential oils through the addition of water to the raw material at a ratio of 6:1 (w/w) and boiling for 1 h. Essential oils were collected through vacuum distillation [11] and the residue was dried at 70 °C for 24 h [12].

*2.2. Dilute-acid hydrolysis*

An autoclave (SANYO MLS-3718L) was used for dilute-acid hydrolysis. Dry OPW was diluted with distilled water at 1:20 and 1:10 (w/v) ratios, while sulfuric acid was added to the mixture at a concentration of 0.5 % (v/v). Mixtures were hydrolyzed at temperatures, time duration and raw material content as specified in (Table 1). Temperatures and reaction times were chosen according to Talebnia *et al* [13], which demonstrated that 116 $^{\circ}$C and 13 min reaction were the optimal conditions for dilute acid hydrolysis of citrus waste. Furthermore, Grohmann *et al* [14] stated that fructose is relatively stable between 100 $^{\circ}$C and 120 $^{\circ}$C in contrast to higher temperatures, whereas the structures of arabinose and galactose are more stable at temperatures above 120 $^{\circ}$C.

*2.3. Pectin recovery*

Centrifugation and filtration followed dilute-acid hydrolysis in order to obtain the supernatant, which was mixed with an equal volume of ethanol (96 % v/v) to precipitate pectin at 25 $^{\circ}$C for 4 h [5]. Consequently, the mixture was centrifuged at 3000 rpm for 30 min. The precipitate was washed five times with ethanol (45 % v/v) followed by drying at 50 $^{\circ}$C [15], while the hydrolyzate was distilled at 80 $^{\circ}$C for the removal of ethanol.

*2.4. Production of cellulase*

*Trichoderma reesei* was employed for the production of cellulase. The yeast was inoculated from -80 $^{\circ}$C glycerol stocks into 1 L shake flasks, containing 200 mL of sterilized fermentation medium. The fermentation medium was composed of (per liter): 40 g wheat bran, 10 g avicel, 3 g peptone, 0.5 g yeast extract, 2 g $(NH_4)_2SO_4$, 0.3 g $CaCl_2 \cdot 2H_2O$, 0.3 g $MgSO_4 \cdot 7H_2O$, 4 g $KH_2PO_4$ and 0.2 g Tween-80. Incubation was performed at 28 $^{\circ}$C, 180 rpm and pH 5.5. Following five days of incubation, the fermentation broth was centrifuged at 3000 rpm for 10 min and the supernatant was filtered through filter paper (0.2 μm pore size). The solution was collected and stored at 4 $^{\circ}$C as crude cellulase liquid (CCL) [6].

*2.5. Microorganism and growth conditions*

The bacterial strain *A. succinogenes* Z130, obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), was used in succinic acid fermentations. The strain was maintained at -80 $^{\circ}$C in glycerol stock cultures and prior to the experiments cells were grown in tryptic soy broth at 37 $^{\circ}$C for 14 h. Batch fermentations were conducted in 100 mL flasks with an initial working volume of 100 mL, which contained 5 g $L^{-1}$ yeast extract and 30 g $L^{-1}$ $MgCO_3$. The hydrolyzate from dilute-acid hydrolysis, obtained following pectin recovery, was used as the only carbon source in fermentations.

Commercial sugars such as fructose, glucose and galactose were studied separately at 10 g $L^{-1}$ initial concentration in batch fermentations. Experiments were carried out in 500 mL flasks with an initial working volume of 500 mL supplemented with contained 5 g $L^{-1}$ yeast extract and 10 g $L^{-1}$ $MgCO_3$. Furthermore, batch fermentations were carried out in a 1 L-bioreactor supplemented with 30 g $L^{-1}$ of glucose, 5 g $L^{-1}$ yeast extract and 30 g $L^{-1}$ $MgCO_3$.

All fermentation processes were carried out at 37 $^{\circ}$C, pH value of 7, 100 rpm and $CO_2$ flow rate 0.5 vvm in duplicates with 13 % (v/v) inoculation volume.

*2.6. Analyses*

*2.6.1. Estimation of succinic acid and by-products concentration*

Succinic acid and other organic acids' formation during fermentation were determined by High Pressure Liquid Chromatography (HPLC). A Shimadzu LC-20AD liquid chromatograph (Shimadzu, UK) equipped with a Shimadzu SPD-20A UV/VIS detector, a Shimadzu SIL-20A HT auto sampler and a CTO-10AS VP column oven was used. The column was eluted isocratically at a rate of 0.6 mL min$^{-1}$ from an organic analysis column (Rezex RHM-Monosaccharide H+ (8 %) column, Phenomenex, USA) with 5 mM $H_2SO_4$ at 50 °C. The injection volume was 20 μL.

*2.6.2. Measurement of reducing sugars*

Orange peel hydrolyzate obtained from dilute-acid hydrolysis (120 μL) were transferred to a 5 mm NMR tube and sodium acetate (50.0 μL, 5.00 mM) was added as internal quantification standard. Deuterium oxide was added up to 500.0 μL final volume. All experiments were prepared twice. $^1$H NMR experiments of fresh prepared solutions were recorded on a 300 MHz Bruker Avance spectrometer, using a pre-saturating pulse for suppressing water absorption peak, relaxation delay 5 s, 2925 Hz spectral window, and 128 scans. Data analysis was performed using MestRenove software.

During acid hydrolysis and fermentation reducing sugars were analyzed by the phenol-sulfuric acid method [16]. This method is based on the phenol-sulfuric acid reaction and is useful for the determination of simple sugars, oligosaccharides, polysaccharides and their derivatives.

*2.6.3. Measurement of enzyme activity*

Cellulase activity was measured using the Filter Paper Unit (FPU) method. The results are given in units of enzyme activity (U), where U is defined as the amount of enzyme that releases 1 μmol of product per minute. The total reducing sugars were estimated by the DNS method [17].

*2.6.4. Elemental analysis*

An inductively coupled plasma mass spectrometer (Thermo X-Series II, Germany) was used for elemental determination of hydrolyzates obtained from dilute-acid hydrolysis (conditions: 109 °C, 10 min, 5 % of dry raw material) and acid/enzyme hydrolysis (109 °C 10 min 5 % of dry raw material/20 IU CCL $g_{raw\ material}^{-1}$). Briefly explained, calibration curves with at least 6 points in the range 5-100 μg L$^{-1}$ were prepared for 21 trace elements: As, Be, Ca, Cd, Co, Cu, Cr, Fe, Li, Mo, Mn, Mg, Ni, P, Pb, Sb, Se, Sr, Ti, Tl and V. The calibration curve with the highest correlation coefficient was used for each element. For the preparation of working standards a multi-analyte calibration standard (Thermo Scientific, Germany) was used. All samples (15 mL) were acidified to 2 % $HNO_3$ and 30 μl of an internal standard mixture of Ga, Lu and In were added prior to analysis. Each sample was analyzed in duplicate and for each duplicate at least 30 mass scans were performed. The concentration of trace elements in the samples was based on monitoring the analyte and its corresponding internal standard. A QC in which a recovery of 80-120 % of spikes and standards was used.

## 3. Results and discussion

### 3.1. Dilute-acid hydrolysis

The dry OPW produced from orange juice production was hydrolyzed with 0.5 % v/v sulfuric acid at a ratio of 1:20 and 1:10 (w/v), at 109 °C and 116 °C for 10 and 20 min. The results obtained from the phenol-sulfuric acid and NMR methods are summarized in Figs. 2A-B. The maximum yield of total carbohydrates liberated from the raw material was achieved at 109 °C for 10 min reaction time with 5 % and 10 % (w/w) of dry OPW, through the addition of 0.5 % (v/v) sulfuric acid (Fig. 2A). According to the results obtained from the NMR method (Fig 2B), which was used for the detection of monosaccharaides and disaccharides in the hydrolyzate, the maximum yield was achieved at 116 °C for 10 min reaction time and 5 % (w/w) of dry OPW. The NMR results also demonstrate that acid hydrolysis carried out using 5 % (w/w) of dry OPW substantially increased the yields compared to the acid hydrolysis performed with 10 % (w/w) of dry OPW.

The presence of hydroxymethylfurfural (HMF), a common inhibitor formed during acid hydrolysis of lignocellulose, in the hydrolyzate was also investigated by NMR as shown in Fig. 3. According to Gunnarsson *et al* [18], concentrations between 0.15 g $L^{-1}$ and 0.35 g $L^{-1}$ of HMF do not impose an inhibitory effect in fermentations. HMF analysis performed in all hydrolyzates measured concentrations below 0.038 g $L^{-1}$, which were approximately an order magnitude lower.

### 3.2. Elemental analysis of OPW hydrolyzates through ICP-MS and investigation of cellulose production

The production of cellulase by *T. reesei* was monitored for 6 days. Using to the FPU method, maximum production of enzymes was achieved following 5 days of cultivation (Fig. 4). Thus, the collection of cellulase was performed at the specific time point for OPW enzyme hydrolysis employed to study the release of elements from the waste. Hydrolyzates obtained from acid hydrolysis as well as a combination of acid and enzyme hydrolysis of OPW, were analyzed by ICP-MS for the presence of metal ions. During acid/enzyme hydrolysis, $Mg^{2+}$ and $Ca^{2+}$ ions were liberated at higher concentrations, as compared to acid hydrolysis (Figs. 5A,C). $Mg^{2+}$ and $Ca^{2+}$ ions serve as important cofactors for *A. succinogenes* fermentations. $Ca^{2+}$ ions are necessary for preserving the fluidity and permeability of the cell membrane, thus facilitating energy and transfer regulation. Furthermore, apart from the positive influence of $MgCO_3$ on succinic acid production, which needs to be supplemented in *A. succinogenes* fermentations for optimal performance, $Mg^{+2}$ ions do not interrupt the stability of the membrane and cell flocculation is not observed [9].

A series of other metal ions were detected at lower concentrations in the hydrolyzates generated (Figs. 5B,D). These elements could be important for succinic acid fermentations [19].

### 3.3. Fermentations

### 3.3.1. Fermentation of commercial sugars to succinic acid

The biomedium containing commercial sugars was supplemented with yeast extract and $MgCO_3$ as described above. Glucose and fructose were fed in fermentations at 10 g $L^{-1}$ (Figs. 6A,B), where the final concentration of succinic acid reached 3.26 g $L^{-1}$ in fructose and 5 g $L^{-1}$ in glucose fermentation respectively. The product yields calculated in these experiments correspond to 0.33 ($g_{succinic acid} g_{fructose}^{-1}$) and 0.57 ($g_{succinic acid} g_{glucose}^{-1}$) for fructose and glucose respectively, while galactose was not fermented by *A. succinogenes*. Apart from succinate,

acetic and formic acids were also formed as by-products in the processes, reaching final concentrations of 1.7 and 3.62 g L$^{-1}$ in fructose fermentations as well as 4.71 and 2.22 g L$^{-1}$ in glucose fermentations respectively.

Furthermore, a batch culture was also performed in a 1 L bioreactor feeding glucose at a concentration of 30 g L$^{-1}$. The maximum concentration of succinic acid achieved was 17.11 g L$^{-1}$ (data not shown) corresponding to a product yield of 0.66 g$_{succinic\ acid}$ g$_{glucose}$$^{-1}$. The results obtained from this work are in agreement to the relevant literature, where succinic acid yields of 0.75 and 0.84 g$_{succinic\ acid}$ g$_{glucose}$$^{-1}$ have been stated [9].

*3.3.2. Succinic acid fermentation of OPW hydrolyzate*

The hydrolyzed OPW was supplemented with yeast extract and MgCO$_3$ and fermented by *A. succinogenes* under anaerobic conditions. Succinic acid and total organic acids yields achieved in fermentations of OPW hyrolyzates are presented in Fig. 7A. The maximum succinic acid yield achieved was 0.88 g$_{succinic\ acid}$ g$_{gtotal\ sugars}$$^{-1}$, which was obtained through fermentation of dilute-acid hydrolyzate obtained at 109 $^o$C for 20 min with 10 % dry OPW. Furthermore, succinic acid yields of 0.77 g$_{succinic\ acid}$ g$_{total\ sugars}$$^{-1}$ and 0.76 g$_{succinic\ acid}$ g$_{total\ sugars}$$^{-1}$ were achieved by fermentation of dilute-acid hydrolyzate obtained at 116 $^o$C for 10 min and 5 % (w/w) raw material as well as 109 $^o$C for 20 min and 5 % (w/w) raw material respectively.

According to the results obtained through NMR, fermentable sugars are liberated at higher concentrations in dilute-acid hydrolysis using 5 % (w/w) of dry OPW as compared to the use of 10 % (w/w) of dry OPW (Fig. 2B). Taking this into consideration, it is expected that a combination of enzyme and dilute-acid hydrolysis performed at 109 $^o$C for 20 min and 5 % (w/w) of dry OPW as well as 116 $^o$C for 10 min and 5 % (w/w) of dry OPW would produce higher amounts of fermentable sugars, compared to the combination of the two pretreatment techniques at 109 $^o$C for 20 min and 10 % (w/w) of dry OPW. Therefore, future investigations of *A. succinogenes* fermentations on OPW hydrolyzates should employ the conditions mentioned above to increase succinic acid productivity. This assumption is also strengthened by the fact that less polysaccharide molecules will be present in a hydrolyzate obtained with a lower raw material concentration. Using a lower initial raw material concentration is beneficial for enzyme activity. The added enzymes would have to convert less polysaccharide molecules into fermentable sugars, thus resulting in higher efficiency for the process of enzymatic hydrolysis. This would ultimately lead to higher product yields, since the amount of soluble sugars produced in the hydrolyzate would correspond to a higher soluble sugar to raw material ratio (g$_{soluble\ sugar}$ g$_{raw\ material}$$^{-1}$).

*A. succinogenes* is predicted to be an industrially important microorganism because of its high efficiency in succinic acid production. Previous studies have shown the production of succinic acid through utilization of various carbon sources (Table 2). The results in this study demonstrate the ability of *A. succinogenes* to valorize OPW to succinic acid with high product yields (0.76 and 0.77 g$_{succinic\ acid}$ g$_{total\ sugars}$$^{-1}$), as compared to the literature (0.115-1.02 g$_{succinic\ acid}$ g$_{total\ sugars}$$^{-1}$). However, the concentrations measured for succinic acid production via hydrolyzate fermentation were 6.13 g L$^{-1}$ for 116 $^o$C, 10 min and 5 % (w/w) of dry OPW as well as 6.17 g L$^{-1}$ for 109 $^o$C, 20 min and 5 % (w/w) of dry OPW (Fig. 7B). However, the low final product concentration achieved can be attributed to the lower initial concentrations of fermentable sugars in the hydrolyzates as compared to other raw materials in the literature. Optimization of OPW pretreatment conditions through a combination of acid and enzyme hydrolysis could lead to higher succinic acid concentrations and improved product yields.

**4. Conclusions**

In this work, a preliminary study for the development of an OPW biorefinery has been presented. Following extraction of essential oils and pectin, the residue was hydrolyzed to fermentable sugars and valorized

towards succinic acid production. The release of metal ions was enhanced with a combination of acid and enzyme hydrolysis. Furthermore, the release of fermentable sugars was maximized in dilute-acid hydrolysis at 109 ºC for 20 min and 10 % (w/w) of dry OPW, 109 ºC for 20 min and 5 % (w/w) of dry OPW and 116 ºC for 10 min and 5 % (w/w) of dry OPW. Similarly, the highest succinic acid production was achieved by fermenting the hydrolyzate obtained through dilute-acid hydrolysis at 109 ºC for 20 min and 5 % (w/w) of dry OPW and 116 ºC for 10 min and 5 % (w/w) of dry OPW. The development of such a biorefinery for the valorization of OPW offers a sustainable and environmental friendly alternative to the current management techniques of orange peel waste.

## Acknowledgements

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**Figure Captions**

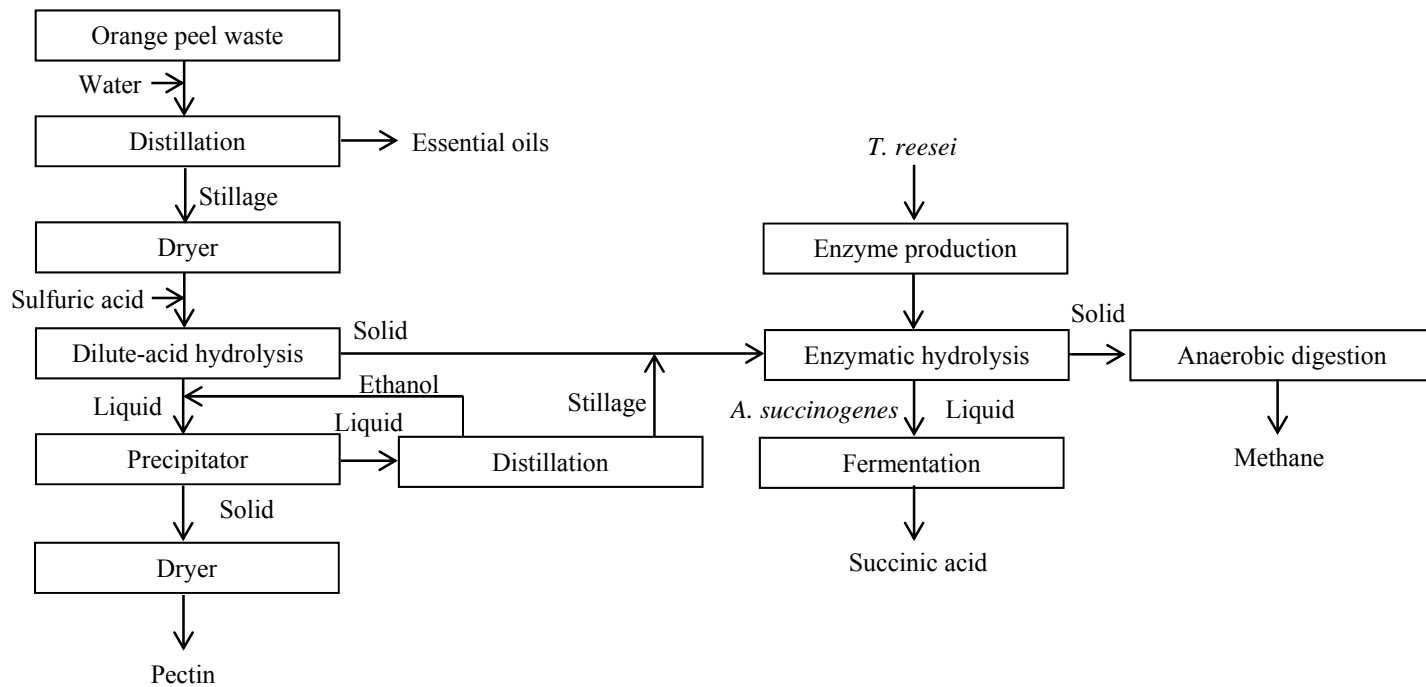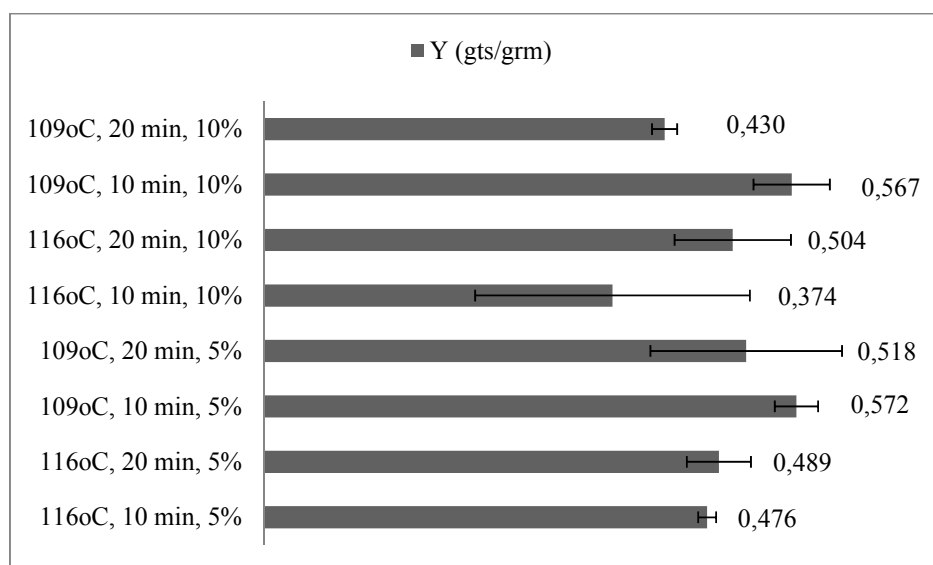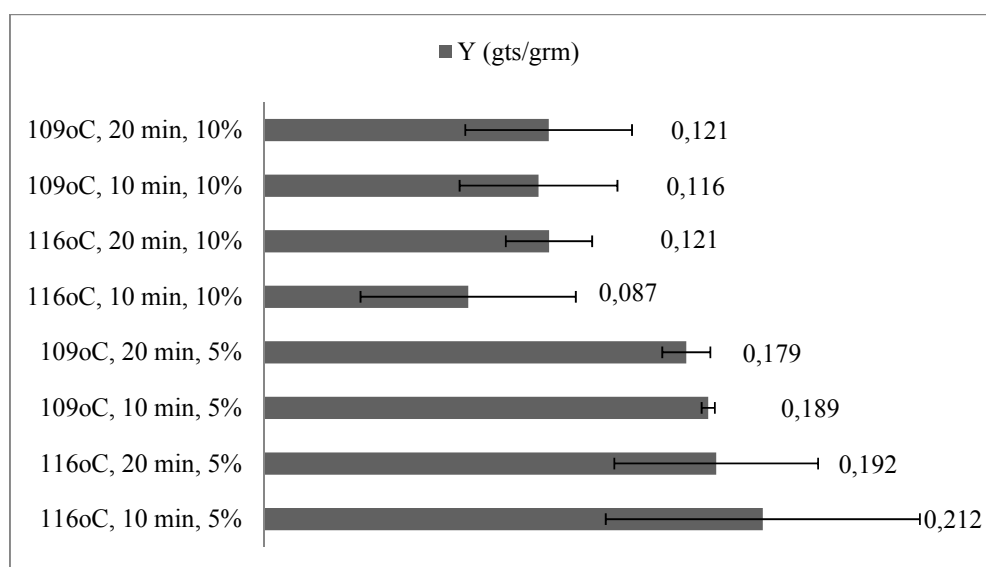**Fig. 1.** Proposed block flow diagram for succinic acid production

**Fig. 2.** Yield of sugars obtained from acid hydrolysis. (A) Phenol-sulfuric acid and (B) NMR methods detection
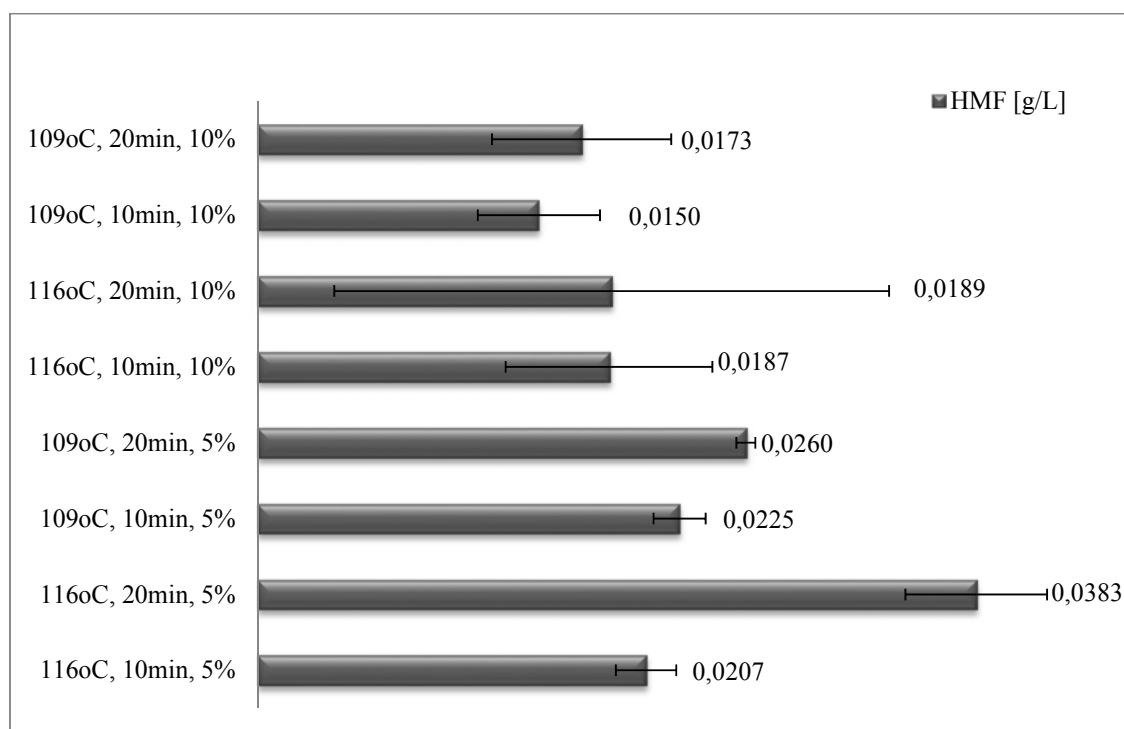


(A)



(B)

ts: total sugars, rm: raw material

**Fig. 3.** Concentration of HMF in dilute-acid hydrolyzates
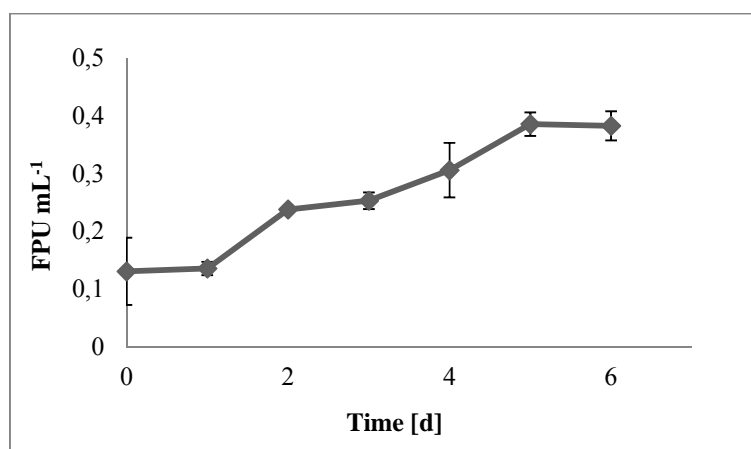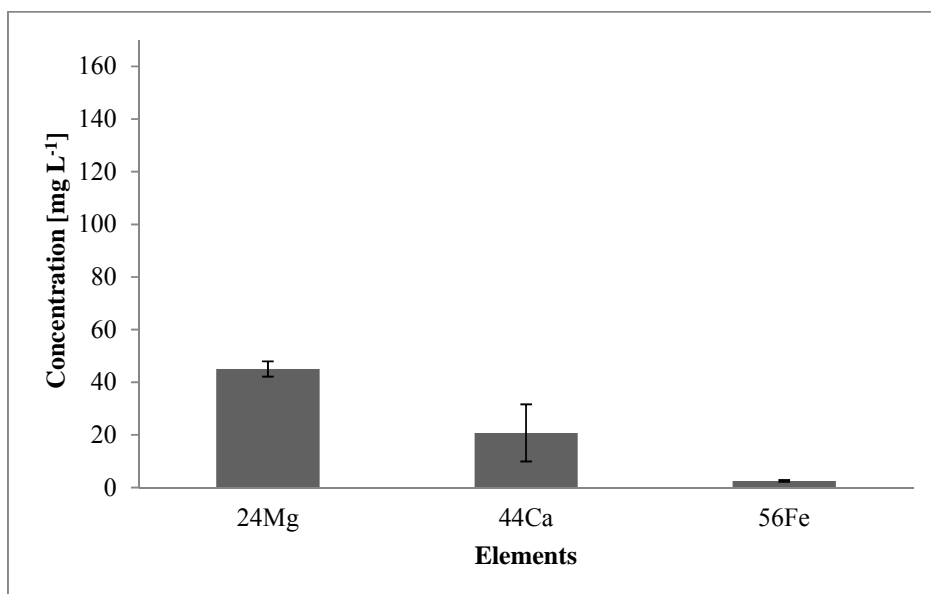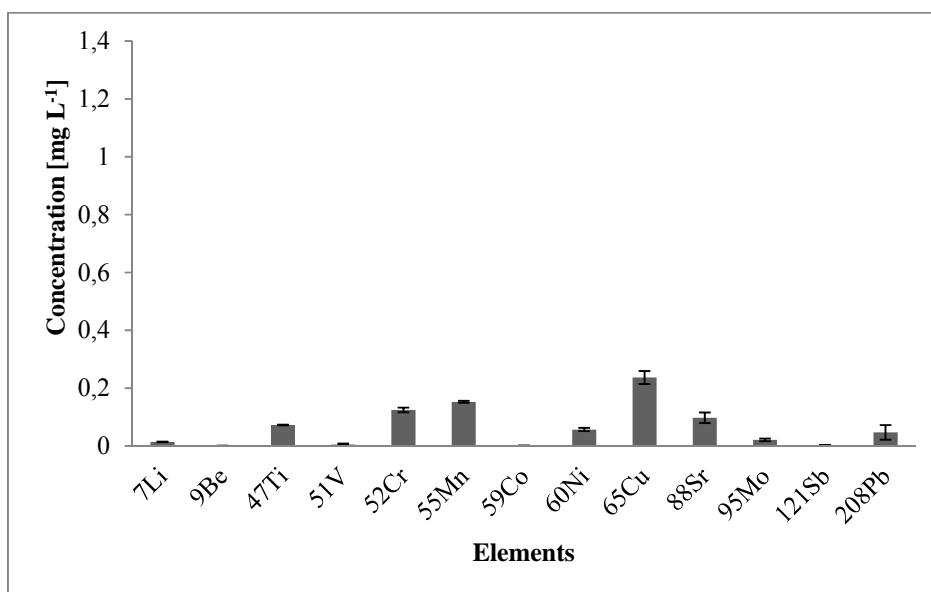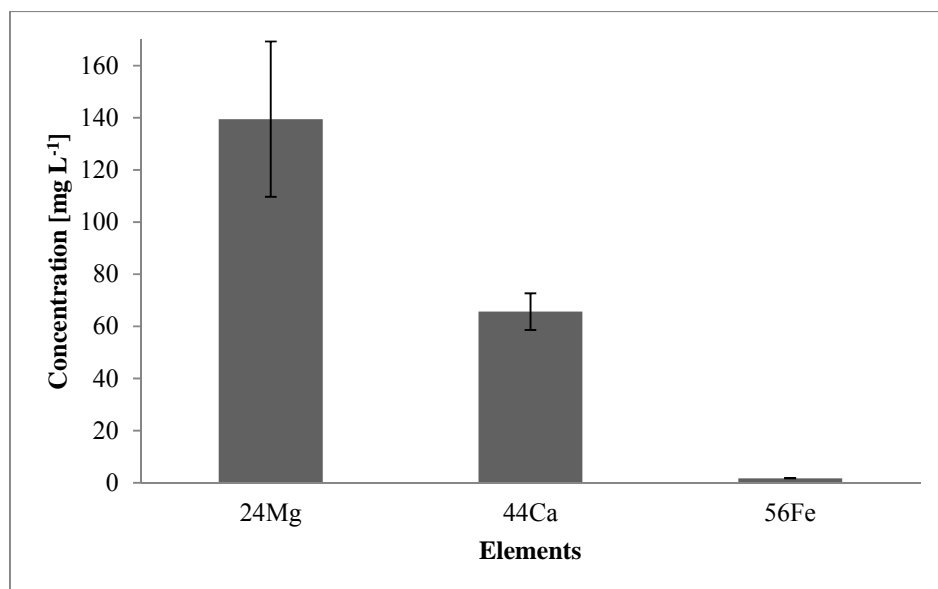
**Fig. 4.** Production of cellulase by *T. reesei*

**Fig. 5.** Concentration of metal ions generated using dilute-acid hydrolysis (A,B) and a combination of acid and enzyme hydrolysis (C,D)



(A)



(B)
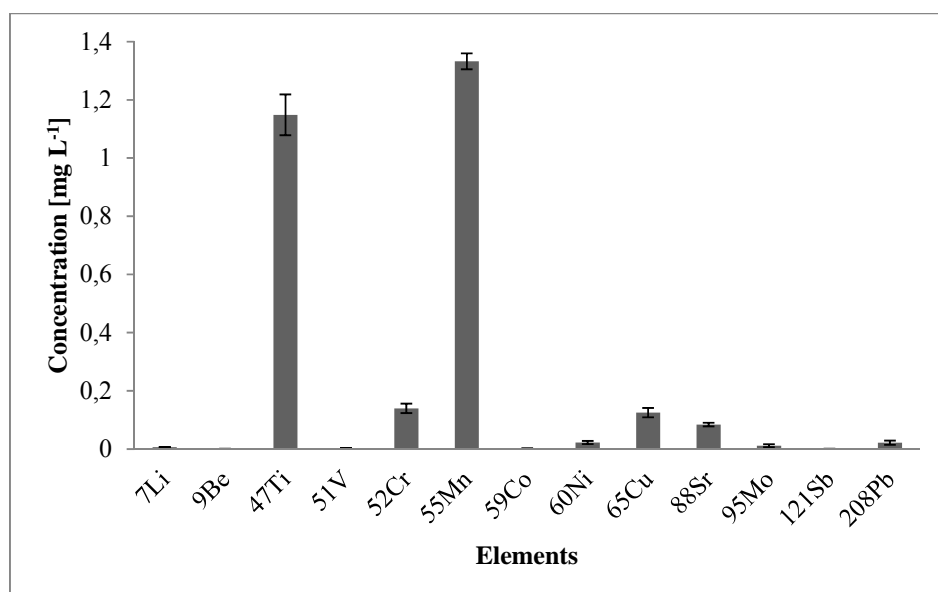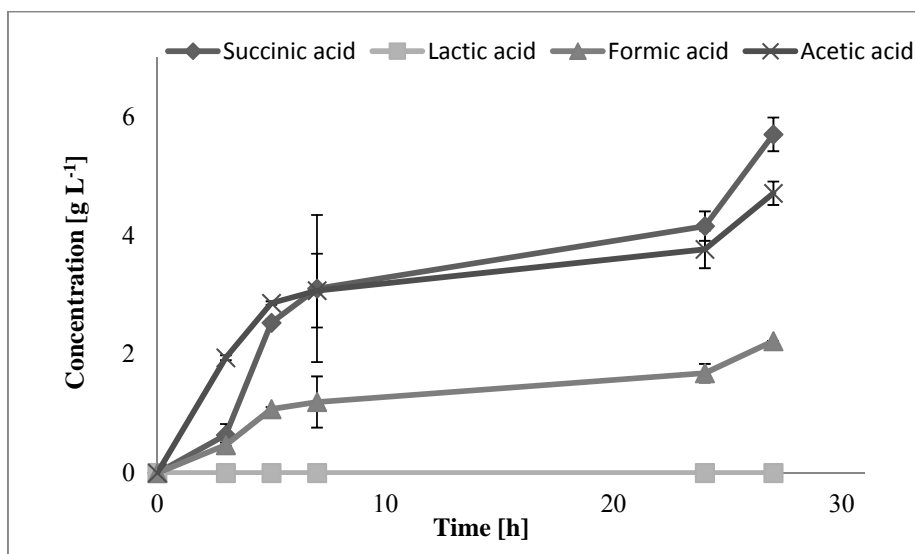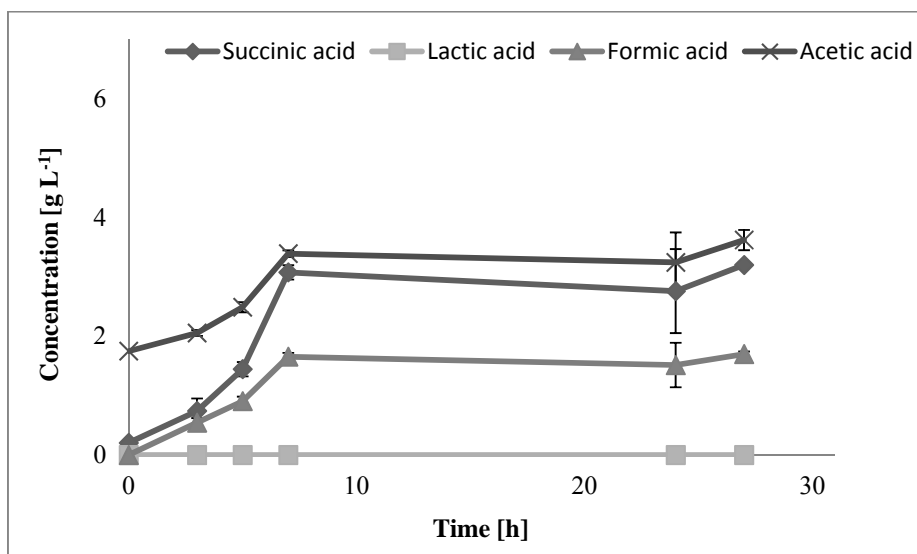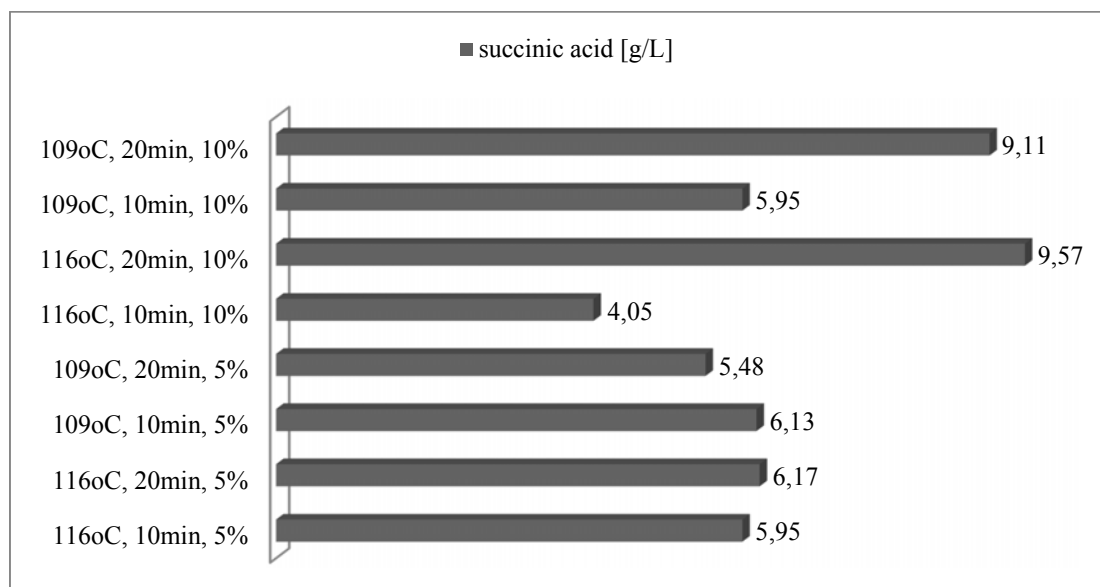
(C)



(D)

**Fig. 6.** Production of organic acids in batch fermentations of *A. succinogenes* with (A) glucose and (B) fructose
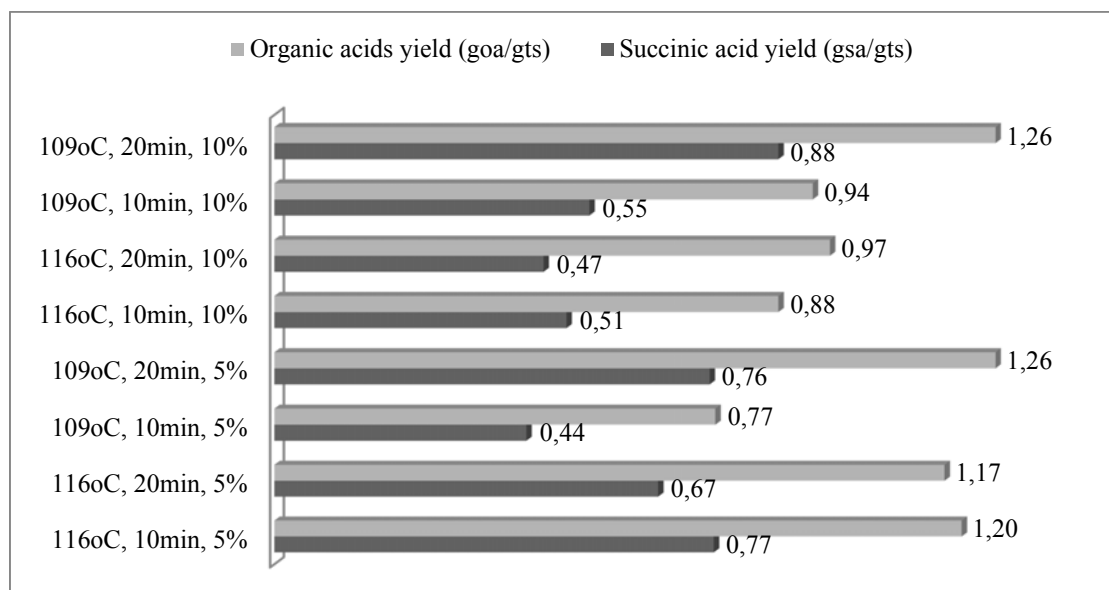


(A)



(B)

**Fig. 7.** Product yields (A) and final succinic acid concentrations (B) achieved in dilute-acid hydrolyzates fermentations



(A)



(B)

oa: organic acids, ts: total sugars, sa: succinic acid

**Tables**

**Table 1.** Dilute-acid hydrolysis conditions

| Temperature [ºC] | Reaction Time [min] | % (w/v) dry raw material |
|---|---|---|
| 109 | 10 | 5 |
| 109 | 20 | 5 |
| 109 | 10 | 10 |
| 109 | 20 | 10 |
| 116 | 10 | 5 |
| 116 | 20 | 5 |
| 116 | 10 | 10 |
| 116 | 20 | 10 |

**Table 2.** Succinic acid bio-production in fermentations utilizing different raw materials

| Raw material | Nitrogen source | Gas supply, Fermentation, Total volume, Working volume | Succinic acid [g L$^{-1}$] | Y [g$_{SA}$g$_{ts}$$^{-1}$] | Reference |
|---|---|---|---|---|---|
| Glycerol | YE (10 g L$^{-1}$) | Anaerobic, fed-batch, bio-reactor 2 L, 1.5 L | 49.62 | 0.64 | [20] |
| Wheat hydrolyzate | YE (5 g L$^{-1}$) / Vit | Anaerobic, batch, bioreactor 1.8 L, 0.5 L | 62.1 | 1.02 | [21] |
| Bread hydrolyzate | BH (200 mg/L FAN) | Anaerobic, batch, bio-reactor 2.5 L, n.d. | 47.3 | n.d. | [22] |
| Cotton stalk hydrolyzate | YE (30 g L$^{-1}$) / Urea (2 g L$^{-1}$) | Anaerobic, batch SSF, flasks 500 mL, n.d. | 63 | 0.64 | [23] |
| Macroalgal hydrolyzate | YE (16.7 g L$^{-1}$) | Anaerobic, batch, bio-reactor 3 L, 1.5 L | 33.78 | 0.63 | [24] |
| Rapeseed meal | YE (15 g L$^{-1}$) | Anaerobic, fed-batch SSF*, bio-reactor 3 L, 1.2 L | 23.4 | 0.115 | [25] |
| Whey | YE (5 g L$^{-1}$) / Pep (10 g L$^{-1}$) | Anaerobic, batch, bio-reactor 2.5L, 1.2L | 22.2 | 0.57 | [26] |
| Dilute-acid hydrolyzate of OPW | YE (5 g L$^{-1}$) | Anaerobic, batch, bottles 100mL, 100mL | 6.17 and 6.13 | 0.76 and 0.77 | Current study |

YE: yeast extract, Vit: vitamins, BH: bread hydrolyzate, Pep: peptone

*Simultaneous saccharification and fermentation