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Autotrophic (C_1-gas) versus heterotrophic (fructose) accumulation of acetic acid and ethanol in *Clostridium aceticum*

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HIGHLIGHTS

- 4.4 g·L^{-1} ethanol production from CO was possible when the pH was not regulated.
- Medium acidification resulted in ethanol production from CO but not from fructose.
- The carbon source affects pH-stimulated solvent production patterns in *C. aceticum.*
- At optimum pH, strong formic acid inhibition occurred during fructose fermentation.

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ABSTRACT

The influence of the carbon source on the metabolism and growth of *Clostridium aceticum* was investigated, supplying either CO or fructose as sole carbon source. The acid and solvent production patterns were determined under either autotrophic or heterotrophic conditions, elucidating the effect of pH on the substrate's bioconversion pattern. The highest maximum specific growth rate was observed with CO, under the organism's optimal growth conditions, reaching 0.052 h^{-1} and an acetic acid concentration of 18 g·L^{-1}. The production of 4.4 g·L^{-1} ethanol was also possible, after medium acidification, during CO bioconversion. Conversely, formic acid inhibition was observed during fructose fermentation under optimal growth conditions. In the latter experiments, it was not possible to stimulate solvent production when growing *C. aceticum* on fructose, despite applying the same medium acidification strategy as with CO, showing the selective effect of the carbon source (autotrophic *vs* heterotrophic) on the metabolic pattern and solventogenesis.

1. Introduction

Bioethanol has been considered a high potential clean transportation fuel alternative to gasoline, that can be produced from sustainable renewable resources. There are two major alternative production routes depending on the feedstock used for obtaining the alcohol. First generation bioethanol production is a commercially mature technology and food-based materials such as sugarcane, sugar beet, corn, and wheat are used as common feedstocks (Sun et al., 2019; de Medeiros et al., 2020; Kennes et al., 2016). However, usage of these food based materials in the production of fuels is criticized for creating food versus fuel competition, associated with increasing market prices of these foods and increasing global food insecurity, causing changes in land use, and deforestation (Kennes et al., 2016; Nanda et al., 2014).

On the other hand, second generation bioethanol production has the potential to minimize these impacts, as lignocellulosic materials, such as agricultural or municipal wastes, wood, straw, grasses, and crop residues are utilized as feedstocks (Groenestijn van et al., 2013). Lignocellulosic biomass may contain up to 30–40% lignin in some cases and valorization of this fraction is an important goal to be reached in order to increase overall product yield and conversion efficiencies (Kennes et al., 2016; Safarian et al., 2020). A possible alternative that allows to use all the lignocellulosic fractions, i.e., cellulose, hemicellulose, and lignin, is biomass gasification, in which the whole feedstock is converted into a gas mixture at high temperature. This gas mixture, known as syngas or synthetic gas, is composed of mainly CO, CO_2 and H_2 gases, with the possible presence of other gases, like CH_4 or nitrogen, and impurities, depending on the gasification conditions (Infantes et al., 2020). Besides

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the gasification of carbon-rich materials, syngas components can also be found in flue gases of some heavy industrial processes (Sun et al., 2019). Carbon monoxide, CO, is also a highly dominant gas in some emissions of steel industries.

Acetogenic microorganisms are able to utilize CO and/or $CO_2 + H_2$ from syngas as sole carbon and/or energy sources and produce organic acids, alcohols and some other chemicals based on the Wood-Ljungdahl pathway (WLP), under anaerobic conditions. Details of the WLP, including the development of the technology, reaction mechanisms and enzymology of the process, naturally occurring products, and recent accomplishments can be found in several review papers (Ragsdale, 2009; Bengelsdorf et el., 2013; Drake et al., 2008; Schiel-Bengelsdorf and Dürre, 2012; Sun et al., 2019).

C. aceticum was the first reported autotrophic acetogen capable of converting carbon dioxide and hydrogen to acetic acid and water in pure culture studies (Wieringa, 1939). However, due to the loss of the strain, further studies were delayed until some spore preparation of the original *C. aceticum* species was found in a culture collection (Braun et al., 1981). Recently, several efforts have been made to study more in details the ability of *C. aceticum* to grow autotrophically on CO and/or $CO_2 + H_2$ in fully automated bioreactor systems (Mayer et al., 2018; Riegler et al., 2019; Arslan et al., 2019). The ethanol production capability of *C. aceticum* was also investigated and optimized very recently and it was shown that lowering the pH of the fermentation medium slightly below 7.0 stimulates the formation of ethanol in this strain. As much as 5.6 $g{\cdot}L^{-1}$ ethanol production was recently reached with different pH regulating strategies from a syngas mixture of $CO:CO_2:H_2:N_2$ (30:5:15:50) (Arslan et al., 2019).

Beyond the characteristic feature of autotrophic growth on syngas components, most members of acetogens have the metabolic flexibility for utilizing a wide range of soluble substrates such as carbohydrates, among others (Fernández-Naveira et al., 2017a; Karekar et al., 2019; Weghoff et al., 2015; Buschhorn et al., 1989). Conversion of carbohydrates to acetic acid in acetogenic bacteria is called homoacetate fermentation and occurs in two steps. In the first step, sugars are oxidized to 2 mol of acetic acid and 2 mol of CO_2 by glycolysis. Following this step, both molecules of CO_2 are further reduced to one additional molecule of acetic acid through the WLP. In total, one mole of sugar is completely fermented to 3 mol of acetic acid according to reactions (1) and (2) (Schuchmann et al., 2016; Huang et al., 2012).

$$Glucose + 4ADP + 4\ P_i \rightarrow 2\ acetic\ acid + 2CO_2 + 4ATP + 2H_2O + 8[H] \quad (1)$$

$$2CO_2 + 8[H] \rightarrow 1\ acetic\ acid + 2H_2O \quad (2)$$

Theoretically, CO_2 reduction to one more mole of acetic acid in the WLP following glycolysis provides the highest known ATP gain and better mass yields in carbohydrate fermentation and only acetogens are natively capable of performing this conversion (Schuchmann et al., 2016; Fast et al., 2015). Therefore, it is important to understand the heterotrophic metabolism of acetogens in order to better understand their potential use in biotechnological applications. Comparison of autotrophic and heterotrophic metabolic patterns of acetogens and their influence on acetogenesis and solventogenesis have hardly been done, except in scarce studies (Fernández-Naveira et al., 2017a).

The present research was undertaken in order to compare the metabolic profile of *C. aceticum* on two different carbon sources, either CO or fructose, as the heterotrophic metabolism of this strain on fructose and possible solventogenesis had not been reported before under controlled conditions, in automated bioreactor systems. Another objective of this research was to study the effect of pH control on the production of metabolites in *C. aceticum* growing on different carbon sources. Several bioreactor studies, with and without pH control, were conducted with both carbon sources in order to elucidate how operational conditions, such as pH, affect the fermentation and bioconversion pattern in *C. aceticum*.

2. Materials and methods

2.1. Microorganism and culture medium

C. aceticum (DSM 1496) was used in all the experiments and was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) in the form of freeze-dried pellets. Initial rehydration of the bacteria was carried out by following the procedure recommended by DSMZ.

The strain was maintained by subculturing in 40 mL working volume serum bottles. Preparation of the serum bottles and growth of seed cultures was done as described previously (Arslan et al., 2019). Autotrophic growth of the strain was also carried out in serum bottles with 40 mL working volume and using CO as carbon source, while fructose was used for heterotrophic growth. The pH value of the inoculum culture medium was set at 8 by using either a 1 M NaOH solution or a 1 M HCl solution. The composition of the liquid culture medium used in all studies with *C. aceticum* was as follows (per liter distilled water): NH_4Cl, 0.20 g; yeast extract, 3 g; KH_2PO_4, 1.76 g; K_2HPO_4, 8.44 g; $MgSO_4$ X $7H_2O$, 0.33 g; $NaHCO_3$, 10 g; L-cysteine-HCl, 0.30 g; Na_2S X $9H_2O$, 0.92 g; rezasurin, 1 mL (from the stock solution of 1 $g{\cdot}L^{-1}$); trace metal solution, 2 mL; vitamin solution, 2 mL.

The composition of the trace metal solution was (per liter distilled water): Nitriloacetic acid, 15 g; $MgSO_4 \times 7\ H_2O$, 30 g; $MnSO_4{\cdot}H_2O$, 5 g; NaCl, 10 g; $FeSO_4 \times 7\ H_2O$, 1 g; $CoSO_4 \times 7\ H_2O$, 1.8 g; $CaCl_2 \times 2\ H_2O$, 1 g; $ZnSO_4 \times 7\ H_2O$, 1.8 g; $CuSO_4 \times 5\ H_2O$, 0.1 g; $KAl(SO_4)_2 \times 12\ H_2O$, 0.2 g; H_3BO_3, 0.1 g; $Na_2MoO_4 \times 2\ H_2O$, 0.1 g; $NiCl_2 \times 6\ H_2O$, 0.25 g; $Na_2SeO_3 \times 5\ H_2O$, 3 mg; $Na_2WO_4 \times 2\ H_2O$, 4 mg.

The composition of the vitamin solution was (per liter distilled water): biotin, 0.025 g; folic acid, 0.025 g; pyridoxine-HCl, 0.050 g; thiamine-HCl, 0.050 g; riboflavin, 0.050 g; nicotinic acid, 0.050 g; D-Ca-pantothenate, 0.050 g; vitamin B12, 0.025 g; *p*-aminobenzoic acid, 0.050 g; lipoic acid, 0.025 g.

2.2. Bioreactor operation

The fermentation studies were carried out in 2L Eppendorf BIOFLO 120 bioreactors (Eppendorf AG, Hamburg, Germany) under strict anaerobic conditions, in batch for the liquid phase (for the heteretrophic cultivation) and with continuous gas supply when feeding a gas phase. The bioreactors were equipped with four baffles, a microsparger and a pH electrode (Mettler Toledo, Columbus, Ohio, USA). The fermentation processes were performed at a temperature of 30°C, which was kept constant by means of either a water jacket or a heating blanket. Mixing was performed with six blade Rushton turbines agitated at 250 rpm. In all studies the initial pH of the fermentation medium was adjusted to 8.0 by using either 1 M HCl or 1 M NaOH solutions.

The bioreactors filled with 1.1 L medium without vitamins, L-cysteine-HCl, Na_2S X $9H_2O$ and fructose were autoclaved at 120°C for 20 min. A 50 mL solution containing L-cysteine-HCl and Na_2S X $9H_2O$ and another 50 mL fructose (36 g) solution were prepared in separate bottles and autoclaved under the same conditions as well. After autoclaving, the medium was flushed with pure aseptic nitrogen for at least 2 h. Following pH adjustment to 8, L-cysteine-HCl, Na_2S X $9H_2O$, fructose and vitamins were added aseptically to the vessels prior inoculation. For inoculation, 120 mL of late-exponential growth phase adapted seed culture was used.

In autotrophic studies fructose was omitted from the medium and replaced with pure CO, that was flushed through the medium for at least 30 min, immediately after nitrogen sparging through the microsparger. The CO gas flow rate entering the reactor was adjusted to 10 mL/min and it was maintained constant during the whole autotrophic experiments by means of a mass flow controller (Aalborg GFC 17, Müllheim, Germany).

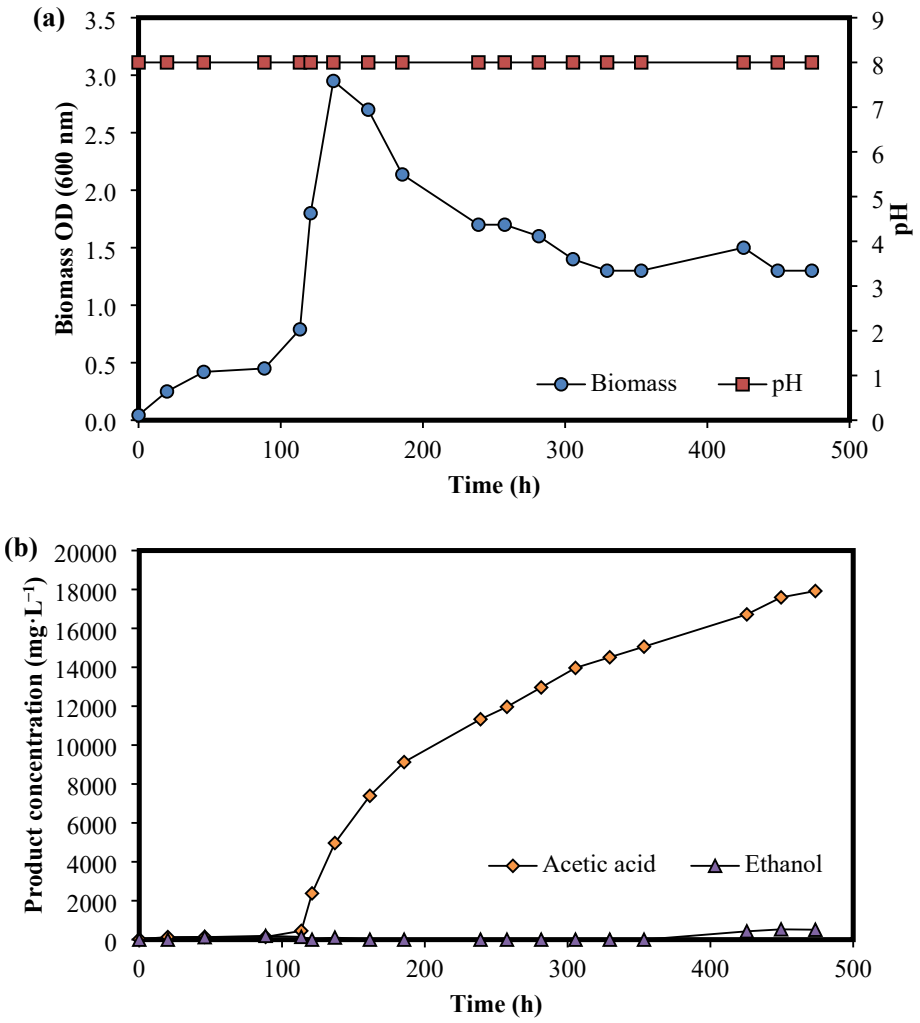

Fig. 1. CO fermentation with pH regulation at 8 (a) biomass concentration (OD value at 600 nm) and pH; (b) metabolites production.

2.3. Analytical methods

2 mL samples were withdrawn daily from the reactor in order to carry out analytical tests. The optical density, used to estimate biomass growth and concentration, was measured with a spectrophotometer (Hitachi, Model U-200, Pacisa & Giralt, Madrid, Spain) at a wavelength of 600 nm.

Fructose, acetic acid and ethanol concentrations were measured on a high performance liquid chromatograph (HPLC) (HP1100, Agilent Co., USA) equipped with a diode array detector and a refractive index detector, operating at 50°C. The possible presence of C4 and C6 acids and alcohols was checked as well. The HPLC samples were first centrifuged (ELMI Skyline Ltd CM 70 M07) at 7000 rpm for 5 min and the supernatant was then filtered through a 0.22 μm filter (Labbox, Barcelona, Spain) before HPLC analyses. The mobile phase used for the HPLC analyses was a 5 mM H_2SO_4 solution, with a flow rate of 0.80 mL/min. 20 μL samples were injected in the Agilent Hi-Plex H Column (300×7.7 mm), which was kept at a constant temperature of 45°C.

Carbon yields, C_M/C_S, were calculated as explained before (Maru et al., 2018). C_M refers the total produced acetic acid carbon and was calculated by multiplying the final acetic acid molar concentration by 2, while C_S refers the total consumed substrate carbon and was calculated by multiplying the total consumed fructose molar concentration by 6.

3. Results and discussion

3.1. CO fermentation with pH regulation and constant pH

In a first experiment, *C. aceticum* was inoculated in the fermentor with continuous CO gas feeding, and at constant pH = 8. This value was kept automatically stable during the whole fermentation process. Fig. 1a and b show, respectively, the bacterial growth and the concentrations of products. A slow growth was first observed at the beginning of the process, which could have resulted from the common inhibitory effect of CO on the initially low biomass concentration. Then, a clear exponential growth phase took place, between about t = 100 h – 137 h, reaching a maximum OD value of 3 at t = 137 h, and with a maximum specific growth rate, μ_{max}, of 0.052 h^{-1}. Afterwards, the biomass OD started to decrease and dropped below 1.5 at t = 300 h; and then it remained roughly constant, around 1.3, until the end of the process.

The start of the exponential growth phase was concomitant with the first detection of acetic acid. The acetic acid concentration remained below 500 $mg{\cdot}L^{-1}$ during the first 100 h fermentation. The highest acetic acid production rate was observed during the exponential growth phase, reaching 0.26 $g{\cdot}L^{-1}{\cdot}h^{-1}$. This acetic acid production went on after the exponential growth phase ended, though with decreasing production rates, dropping gradually from 0.1 to 0.02 $g{\cdot}L^{-1}{\cdot}h^{-1}$ at the end of the process, and reaching a final product concentration of 18 $g{\cdot}L^{-1}$, after 475 h fermentation. Some ethanol production was observed as well, but only after almost 400 h, to finally reach around 500 $mg{\cdot}L^{-1}$

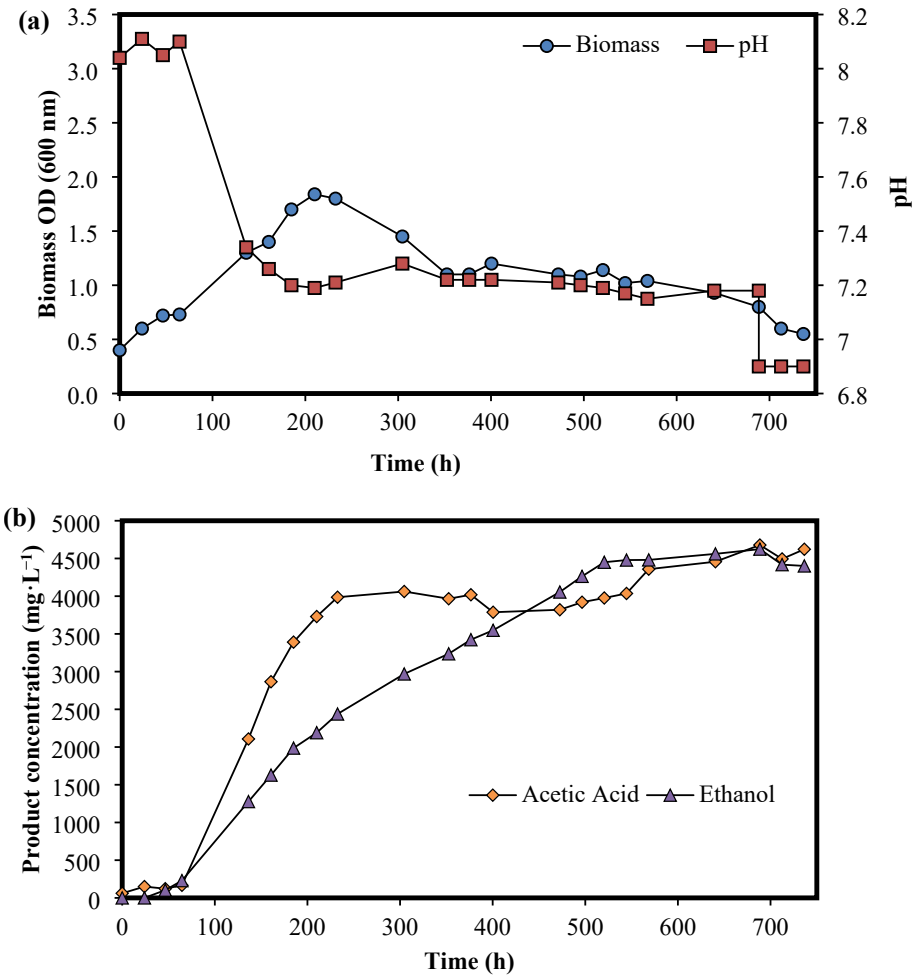

Fig. 2. CO fermentation without pH regulation (a) biomass concentration (OD value at 600 nm) and pH; (b) metabolites production.

in the fermentation medium.

3.2. CO fermentation without pH regulation and with medium acidification

A subsequent CO conversion experiment was conducted in order to evaluate the effect of pH on metabolite production and on the biomass growth of *C. aceticum*. This experiment was started under optimal conditions, at pH = 8, but after inoculation, pH regulation was switched off and the pH value was allowed to drop freely, depending on the nature of the products and their concentrations. Fig. 2a and b show the biomass growth, products formation, and pH fluctuations during the fermentation process. The initial bacterial growth was slow, similarly as in the previous experiment. Besides, a longer exponential growth phase was observed, which took place between t = 65 h – 210 h of the fermentation process but with a much lower specific growth rate, μ_{max}, of 0.006 h^{-1}, than in the previous case. This could certainly be related to the fast pH drop as a result of acetic acid production. Initially, slow production of acetic acid took place, before the exponential growth phase, and then a much higher acid production rate was observed, leading to a fast pH drop from 8 to 7.3, simultaneous to a sharp increase of the acetic acid concentration up to 2.1 $g{\cdot}L^{-1}$ (Fig. 2a and b). The optimum pH range of *C. aceticum* for cell growth was reported to be between 8 and 8.5 and it was also reported that at pH values below 7 or above 10.5 generally no growth is observed (Wieringa, 1939; Braun et al., 1981). In the present study, even though bacterial growth continued while the pH decreased down to around 7.2, together with acetic acid production, the maximum OD reached a value of only 1.8, which is significantly lower than in the previous experiment at constant optimal pH. As expected, suppressing pH regulation led to a drop of the fermentation pH and slowed down the bacterial growth, leading to a longer and slower exponential growth phase with a significantly lower final cell density. After the exponential growth phase, the biomass OD decreased to around 1.0 and remained close to this value until the end of the process. Biomass decay may, among others, be attributed to the low pH value.

The acetic acid production rates were also lower compared to the previous study due to the lower bacterial growth and the highest production rate was observed during the exponential growth phase, corresponding to 0.03 $g{\cdot}L^{-1}{\cdot}h^{-1}$. The acetic acid concentration increased slowly and reached a stable value, around 4 $g{\cdot}L^{-1}$, at the end of the growth stage, causing a pH drop to 7.19. This acetic acid concentration is significantly lower than when maintaining the medium at constant, near optimal, pH. Ethanol first appeared in the fermentation medium at t = 46 h while the medium's pH was still around 8, but it was detected in only minor amounts. Afterwards, with the pH value fastly decreasing from 8 to 7.3, a high ethanol production was observed, simultaneous to acetic acid accumulation and bacterial growth. The ethanol production rates were always lower than the acetic acid production rates during the exponential growth phase; however, the highest ethanol production rates, of around 0.015 $g{\cdot}L^{-1}{\cdot}h^{-1}$, were still observed during this stage as a result of the high active bacterial concentration. Ethanol continued accumulating in the fermentation medium after acetic acid had reached a stable, near constant, concentration. Later, some small decrease was detected, and when ethanol production leveled off, after 520 h, some very low increase in acetic acid concentration was still observed. Considering this production pattern, it is hypothesized that after the

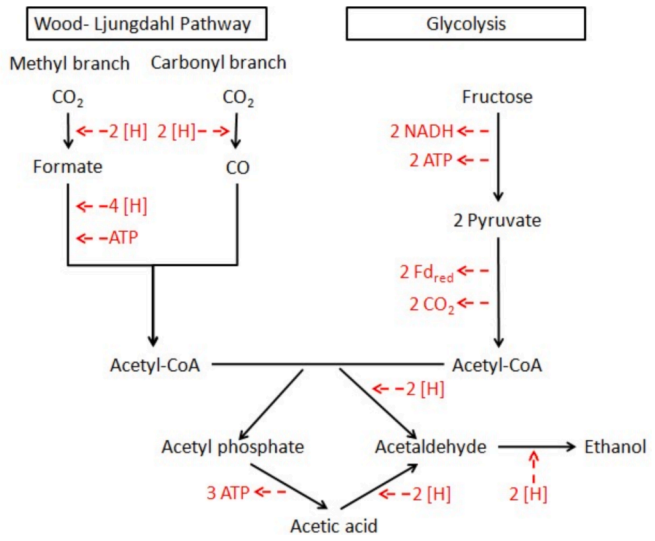

Fig. 3. WLP and glycolysis in acetogens (Modified from Bengelsdorf et al., 2013; Schuchmann et al., 2016).

exponential growth phase non-growth coupled acetic acid formation continued with lower acid production rates, while part of the produced acid was simultaneously assimilated and converted to ethanol, resulting in both production and consumption of the acid. In a recent study with continuous syngas feeding ($CO/CO_2/H_2/N_2$), rather than pure CO, and with *C. aceticum*, lowering the pH also stimulated solvent production (Arslan et al., 2019). In that study, high amounts of ethanol were only observed when the pH of the medium was decreased below neutral conditions, and a clear switch from acidogenesis to solventogenesis was also shown, slightly below pH 7.0, with a decrease in acetic acid concentration concominant to increases in ethanol concentrations. Hence, it was concluded that rather than a direct conversion of C_1 gases to alcohol, ethanol production took mainly place through the re-assimilation of acetic acid at low pH values. It was also reported before, by other authors, that rather than the direct reduction of acetyl-CoA to ethanol, conversion of acetic acid to ethanol through the aldehyde:ferredoxin oxidoreductase (AOR) (Fig. 3) route is energetically more favorable in acetogens (Mock et al., 2015). According to these results, in order to analyze any further possibility of ethanol accumulation, after t = 690 h, the fermentation pH was decreased artificially to 6.9, by using 1 M HCl, as a pH value slightly below neutral was recently reported as the most suitable average pH value for stimulation of solvent production in *C. aceticum* (Arslan et al., 2019). Nonetheless, lowering the fermentation pH to 6.9 only resulted in faster bacterial decay, while any possible improvement in ethanol production was not observed, which was most probably also a consequence of the low biomass concentration. At the end of the experiment, at t = 690 h, the final concentrations of acetic acid and ethanol had reached 4.7 $g{\cdot}L^{-1}$ and 4.6 $g{\cdot}L^{-1}$, respectively, highlighting the unfavorable effect of the applied pH conditions on acetogenesis and the solventogenic pattern leading to considerable ethanol accumulation.

There are few known acetogenic bacteria capable of growing on CO and convert this gas to acids and their corresponding alcohols (Köpke

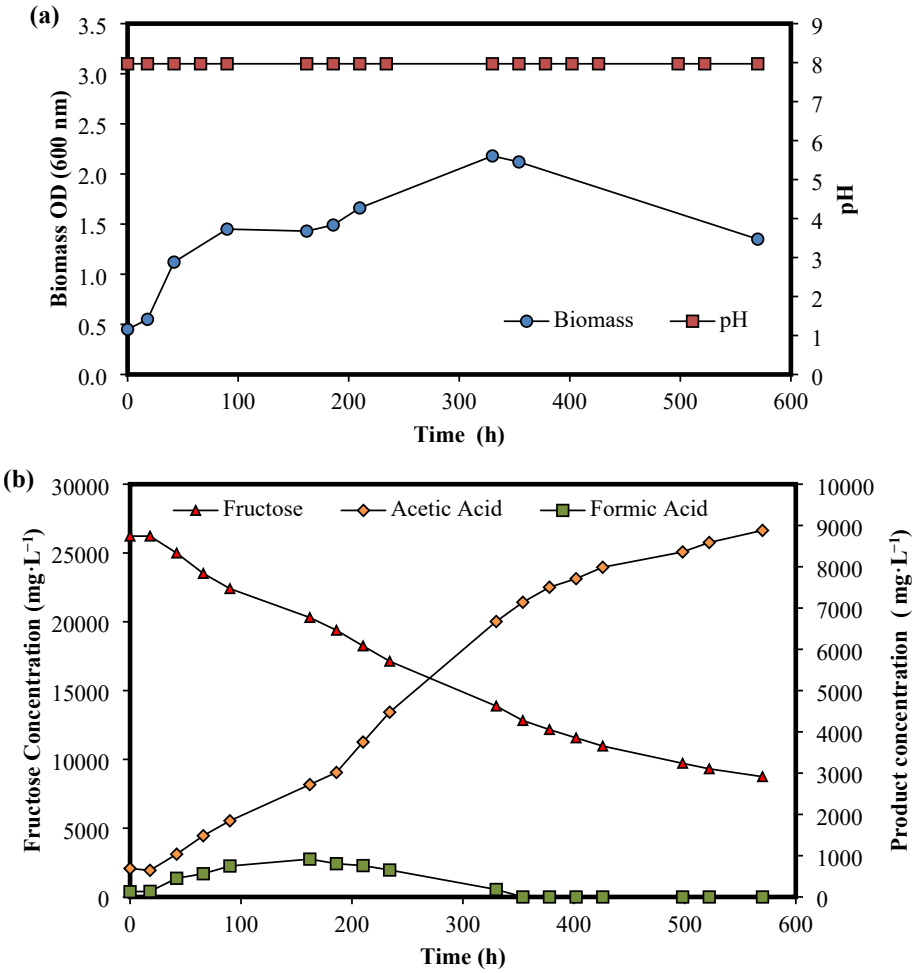

Fig. 4. Fructose fermentation with pH regulation at 8 (a) biomass concentration (OD value at 600 nm) and pH; (b) metabolites production and fructose consumption.

et al., 2011). Stimulation of the solventogenic phase and the shifting from acetate to ethanol production usually occur under inhibitory or stress growth conditions, such as nutrient limitation (Saxena and Tanner, 2011) or below-optimal pH values (Abubackar et al., 2012; Fernández-Naveira et al., 2019). pH values below optimal generally stimulate solventogenesis, while they inhibit or slow down acetogenesis and biomass growth (Groenestijn van et al., 2013). However, lower pH values are also causing fast bacterial decay and lower bacterial densities resulting in the slowing down of ethanol accumulation rates. As already mentioned, in a previous study with a syngas mixture ($CO:CO_2:H_2:N_2$, 30:5:15:50), it was shown that *C. aceticum* was capable of producing ethanol; however, the start of ethanol production was only possible at pH values slightly below 7, which is in turn inhibitory for bacterial growth. Pure CO was reported to be a better carbon and electron source compared to the $CO_2 + H_2$ or syngas mixtures, e.g., $CO + CO_2 + H_2$. CO was found to allow higher bacterial growth rates and improved production of reduced metabolites like ethanol and 2,3-butanediol at higher amounts (Hermann et al., 2020; Mayer et al., 2018). In the present study, the growth of *C. aceticum* on CO was faster and the maximum biomass OD was higher compared to what was recently reported with syngas under optimal conditions (Arslan et al., 2019). In this experiment, ethanol appeared in the fermentation medium already when the pH value was still 8, and the highest ethanol production rates were observed during the exponential growth phase, while pH was then fluctuating between 7.35 and 7.2. At the end of the exponential growth phase, at t = 210 h, 50% percent of the total final ethanol concentrations had already accumulated in the fermentation broth. However, it took another 480 h for the ethanol concentration to reach its maximum value of 4.6 $g{\cdot}L^{-1}$, which did not increase further, as a result of the low bacterial concentration. Arslan et al. (2019) reported, the highest, maximum, ethanol concentration of 5.6 $g{\cdot}L^{-1}$, produced from syngas, after a process time of 710 h with this strain by applying a pH shifting strategy which consisted of controlled pH shifts between optimal growth pH values and low pH values, and which was found to be optimal for solventogenesis (Arslan et al., 2019). It is interesting to highlight that, despite some slight differences, the behavior of *C. aceticum* observed here with CO is rather similar as with syngas (Arslan et al., 2019), though differences are observed between the autotrophic growth and the heterotrophic growth studied and described hereafter. It is also worth to highlight that in the few acetogenic bacteria known so far to produce alcohols from CO, CO_2 or syngas, solventogenesis is stimulated at low, acidic pH values, often around 5 or lower (Abubackar et al., 2016; Fernández-Naveira et al., 2017b), while pH for solventogenesis in *C. aceticum* takes place around neutral pH values and slightly below.

3.3. Fructose fermentation with pH regulation and constant pH

Acetogens are able to fully metabolize and completely convert carbon substrates to end products, often under either autotrophic or heterotrophic conditions. Mixotrophy and the simultaneous utilization of both organic and inorganic carbon sources is frequently also possible. Since *C. aceticum* was reported to grow efficiently on fructose (Braun and Gottschalk, 1981), this sugar was chosen as the carbon source for a new experiment, carried out to assess the behavior of *C. aceticum* in a stirred tank reactor (STR) at optimum growth pH of 8. Fig. 4a and b show the heterotrophic growth profile and metabolites production. The experiment was started with an initial fructose concentration of 27 $g{\cdot}L^{-1}$ as this concentration was considered to be sufficient for bacterial growth and to assess the metabolites production profile. After reactor inoculation, the strain started growing and reached an OD of 1.5, after t = 90 h, with a maximum specific growth rate, μ_{max}, of 0.029 h^{-1}. This rate appears to be slightly lower on fructose than on CO, while higher or similar maximum specific growth rates are more usual when acetogenic bacteria grow on carbohydrates rather than on C_1 gases (Fernández-Naveira et al., 2017a, 2017b). After t = 90 h, a second, slower, growth phase was observed between t = 180 and 330 h. Finally, a maximum biomass OD of 2.2 was reached at t = 330 h, which later started slightly decreasing. A rather constant linear consumption of the carbon source, fructose, was observed and a similar production pattern was found for the main product, acetic acid. The highest fructose consumption rate was found to be 0.06 $g{\cdot}L^{-1}{\cdot}h^{-1}$, during the first exponential growth phase, while highest acetic acid production rates were observed during second growth phase, after 180 h of bioconversion process, and reached 0.03 $g{\cdot}L^{-1}{\cdot}h^{-1}$. Formic acid was also transiently detected in the fermentation medium together with acetic acid, during the first 330 h of the study, increasing up to a maximum concentration of 0.9 $g{\cdot}L^{-1}$ at t = 160 h. Afterward, its concentration decreased down to trace amounts, within 170 h after reaching its maximum value. This same behavior was observed with *C. carboxidivorans*, in which a transient production of formic acid was detected in that species when grown on glucose at a constant near optimal pH (Fernández-Naveira et al., 2017a), while this was not found in that species when grown on CO or syngas. The appearance of formic acid and acetic acid were basically simultaneous, both in *C. aceticum* grown on fructose (this study) and in *C. carboxidivorans* grown on glucose (Fernández-Naveira et al., 2017a). Thus, accumulation of formic acid, even transiently, is more common during heterotrophic growth on sugars than in autotrophic acetogenic growth. At the end of the 570 h fermentation process, the final remaining fructose concentration was 8.7 $g{\cdot}L^{-1}$ and acetic acid was the only fermentation product accumulating in the medium, with a final concentration around 8.8 $g{\cdot}L^{-1}$.

It was mentioned before that due to the low solubility of the syngas components in water, C_1-gas fermentation may suffer from low productivities as a consequence of poor cell growth. According to some previous studies carried out with different acetogenic microorganisms, growth on sugar based carbon, rather than syngas components, sometimes resulted in more dense cultures (Liu et al., 2015). It is also known that the doubling time for growth of *C. aceticum* with H_2 and CO_2 (20–25 h) is higher than with fructose (8 h) (Braun et al., 1981). However, in this study, the maximum specific growth rate and maximum bacterial density were lower compared to the experiment performed with CO under the same conditions. A possible explanation could be the accumulation of formic acid in the fermentation broth which was reported in a previous study with *C. carboxidivorans* grown on glucose as well. It was concluded that formic acid could lead to an inhibition effect, triggering acidic crash in that species (Fernández-Naveira et al., 2017a). As can be seen in Fig. 4a and b, once the formic acid concentration reached 750 $mg{\cdot}L^{-1}$, biomass growth slowed down significantly and nearly stopped for a few hours. Later, when the formic acid concentration decreased below 750 $mg{\cdot}L^{-1}$, after t = 186 h, the biomass continued growing again. Given this pattern of biomass OD and metabolite formation it could be inferred that formic acid accumulation inhibited biomass growth in *C. aceticum* as well.

The autotrophic and heterotrophic metabolic pathways of acetogens are shown in Fig. 3. During glycolysis, fructose is converted to 2 mol of acetyl-CoA, 2 mol of CO_2, 2 mol ATP, and excess reducing equivalents (e.g. NADH, Fd_{red}). These reducing equivalents are used in the WLP to fix 2 mol of CO_2 into 1 more mole acetyl-CoA. Even though autotrophic and heteretrophic metabolisms of acetogens have been investigated and reported, the correlation of the pathways is species dependent (Liu et al., 2015; Maru et al., 2018).

Fructose fermentation by *C. aceticum* resulted in formic acid accumulation in the culture medium, which is an indicator of WLP activity since formic acid is an intermediate product of the WLP's methyl branch (Fig. 3). Theoretically, the amount of reducing equivalents synthesized by glycolysis is exactly the amount required to fix 2 mol of CO_2 to acetyl-CoA through the WLP. However, when the flux between glycolysis and WLP is not established, excess reducing equivalents are oxidized by hydrogenase activity which results in H_2 release. In the first step of the WLP's methyl branch, 2[H] are required for the conversion of CO_2 to formic acid, later 4 more [H] are used for complete conversion to acetyl-CoA. Formic acid accumulation in the fermentation medium is an

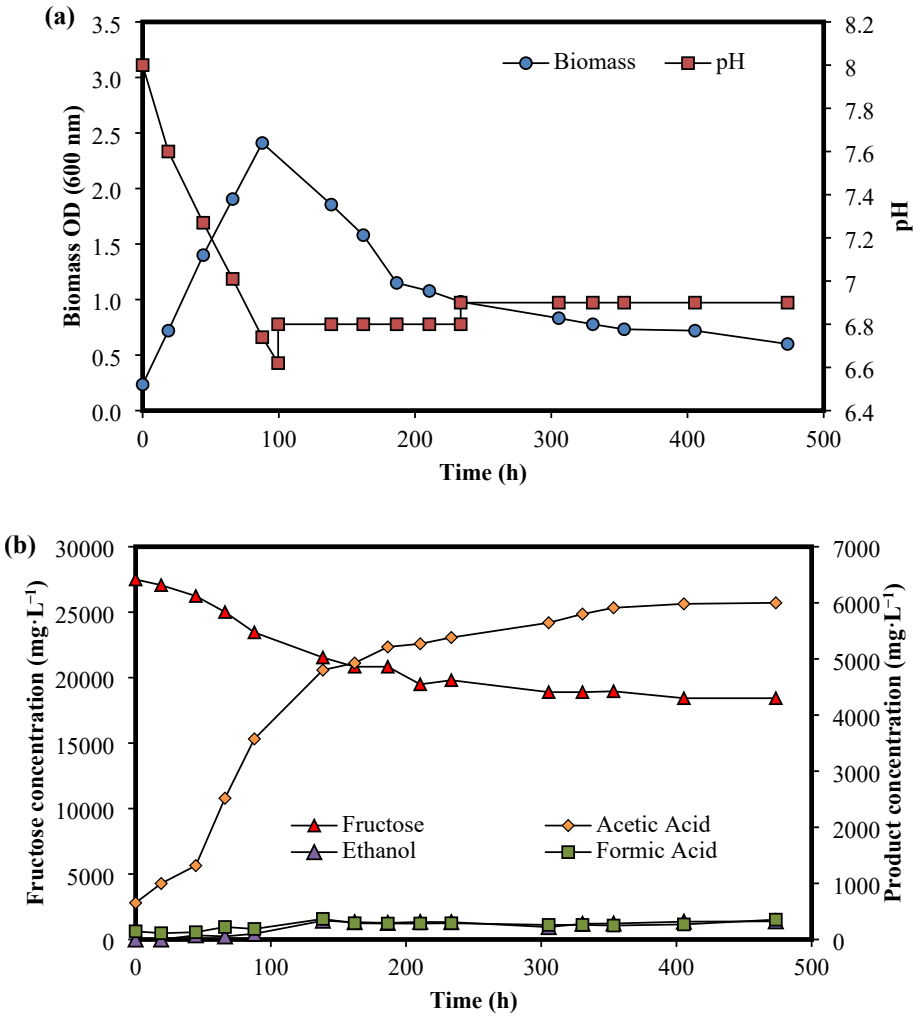

Fig. 5. Fructose fermentation without pH regulation (a) biomass concentration (OD value at 600 nm) and pH; (b) metabolites production.

evidence of the imbalance in fluxes of reducing equivalents between the two pathways. Fast formic acid accumulation in the fermentation medium was also reported before in *C. aceticum* when the WLP metabolism was purposely disturbed (Mayer et al., 2018). In sugar fermentation by acetogenic bacteria, a possible WLP disruption could be explained by a phenomenon called carbon catabolite repression, a regulatory mechanism in which bacteria repress the consumption of a secondary carbon source in the presence of a preferred carbon source. This phenomenon is indicating that in the presence of fructose, WLP genes might be down regulated causing a disruption of CO_2 consumption by the WLP (Fast et al., 2015). Considering the above information, it could be concluded that, in the present study, the presence of fructose in the fermentation medium repressed the autotrophic metabolism in *C. aceticum*, resulting in formic acid accumulation which created an inhibition of bacterial growth and prevented further fructose consumption and metabolite formation. The repressing effect from the presence of sugars on CO_2 assimilation through the WLP in homoacetogens was reported in *Blautia coccoides* as well, showing that higher glucose concentrations are lowering the autotrophic ability of the bacteria (Liu et al., 2015). Maximum carbon yields C_M/C_S, or the ratio of carbon in produced metabolites to carbon in carbohydrate consumed, during the conversion of sugar are 67% at best due to the fact that one-third of all carbon in the sugar is lost to CO_2. Moreover, this ratio is actually even lower than 67% because of the carbon fixation in cell mass or the maintenance needs of the bacteria (Jones et al., 2016; Maru et al., 2018). Thus, it could be concluded that the observed C_M/C_S ratios, higher than 67%, can be considered as a successful mixotrophy. Final C_M/C_S derived in the present study was only 48% when considering acetic acid as the only product of the process. Even though formic acid formation is proving the occurrence of autotrophic metabolism in *C. aceticum*, it was strongly repressed by the presence of fructose. The CO_2 gas, that was not reassimilated through the WLP was lost, resulting in poor carbon yields. Depending on the C_M/C_S values, poor mixotrophic behaviors were characterized before in some other acetogenic bacteria such as *Acetobacterium carbinolicum, Blautia producta* and *Eubacterium limosum* growing on a preferred carbohydrate while some others like *Clostridium drakei, Clostridium magnum* and *Clostridium scatologenes* were shown to perform much better mixotrophy (Maru et al., 2018).

3.4. Fructose fermentation without pH regulation and with medium acidification

In order to determine the impact of pH on the metabolism of *C. aceticum*, depending on the carbon source, an additional experiment was performed with fructose, without pH adjustment. This experiment was designed and initiated as described in section 3.2. pH was adjusted to 8 at the beginning and, after inoculation, pH adjustment was stopped, so that its value could fluctuate depending on the products formed and their concentrations. Fig. 5a and b show the growth of *C. aceticum* and the formation of fermentation products. During the first 88 h, the biomass OD increased up to a maximum value of 2.4, with a μ_{max} of 0.017 h^{-1}. This growth coincided with acetic acid accumulation, up to 4

Table 1
Summary of the results.

	CO fermentation at pH 8	CO fermentation without pH control	Fructose fermentation at pH 8	Fructose fermentation without pH control
OD_{600} max.	3 (137 h)	1.8 (210 h)	2.2 (330 h)	2.4 (88 h)
μ_{exp}	0.052 h^{-1}	0.006 h^{-1}	0.029 h^{-1}	0.017 h^{-1}
Maximum acetic acid formation rate	0.26 $g{\cdot}L^{-1}{\cdot}h^{-1}$	0.03 $g{\cdot}L^{-1}{\cdot}h^{-1}$	0.03 $g{\cdot}L^{-1}{\cdot}h^{-1}$	0.06 $g{\cdot}L^{-1}{\cdot}h^{-1}$
Final acetic acid concentration	18 $g{\cdot}L^{-1}$	4.7 $g{\cdot}L^{-1}$	8.8 $g{\cdot}L^{-1}$	6 $g{\cdot}L^{-1}$
Final ethanol concentration	0.5 $g{\cdot}L^{-1}$	4.6 $g{\cdot}L^{-1}$	–	0.325 $g{\cdot}L^{-1}$
Maximum formic acid concentration	–	–	0.9 $g{\cdot}L^{-1}$	0.35 $g{\cdot}L^{-1}$
Final fructose concentration	–	–	8.7 $g{\cdot}L^{-1}$	18.4 $g{\cdot}L^{-1}$
C_M/C_S	–	–	48%	60%
Maximum fructose consumption rate	–	–	0.06 $g{\cdot}L^{-1}{\cdot}h^{-1}$	0.07 $g{\cdot}L^{-1}{\cdot}h^{-1}$

$g{\cdot}L^{-1}$, and a pH decrease from 8 to 6.7. The bacterial OD reached its maximum value when the pH dropped to approximately 6.7 at t = 88 h. The pH value was then slightly adjusted and set at 6.8, between t = 100 and 230 h, and later, it was further increased to 6.9 and controlled at these values in order to avoid any further pH drop which could cause a fast bacterial death and inhibition. Low amounts of ethanol in the fermentation medium first appeared after 50 h when the pH of the medium was around 7.3. The ethanol concentration peaked at 330 $mg{\cdot}L^{-1}$, at t = 140 h, when the pH value was 6.8 and no more substantial increase in ethanol production was observed for the rest of the process. Formic acid concentrations, between 100 and 300 $mg{\cdot}L^{-1}$, were detected during the process and no evident inhibition effect was observed on biomass growth or metabolite production, that could be related to formic acid formation. About 400 h after fructose consumption and metabolites production stabilized and at the end of the 475 h process time, the concentrations of fructose, acetic acid, ethanol and formic acid in the fermentation medium were 18.4 $g{\cdot}L^{-1}$, 6 $g{\cdot}L^{-1}$, 325 $mg{\cdot}L^{-1}$ and 350 $mg{\cdot}L^{-1}$, respectively.

In comparison to the previous experiment, with fructose and pH maintained at 8, although some formic acid was observed in the fermentation medium, which could be interpreted as the occurrence of autotrophy (i.e., CO_2 coming from glycolysis), its concentration never reached inhibitory levels during the uncontrolled pH study. Bacterial growth was slower solely as a result of the pH drop, and while specific bacterial growth rates were lower than in the previous experiment, the maximum biomass OD was observed to be marginally higher. The maximum acetic acid production and maximum fructose consumption rates were higher than in the previous study as well and were observed to reach 0.06 and 0.07 $g{\cdot}L^{-1}{\cdot}h^{-1}$, respectively, during the initial growth phase.

Once the pH was set at 6.8, a fast bacterial death was observed, resulting in low final metabolites concentrations, and only 33% of the initial fructose concentration (27 $g{\cdot}L^{-1}$) was consumed during the process. However, a better C_M/C_S ratio, of 60%, was observed, showing more efficient carbon fixation in the biomass and metabolites under uncontrolled pH conditions. It is suggested that the reason for the less efficient utilization of fructose in the previous experiment was possibly the increased maintenance need caused by the inhibitory effect of formic acid accumulation.

As mentioned before, metabolite formation during heterotrophic growth of *C. aceticum* is dependent on the availability of sufficient amount of reducing equivalents. As can be seen in Fig. 3, reduction of acetyl-CoA or acetic acid into ethanol requires 4 more [H]. pH drop clearly stimulated ethanol production when *C. aceticum* was growing on CO; however, the same effect was not observed in fructose fermentation. A possible explanation for this difference could be the lack of reducing equivalents. The same behavior was reported in *C. carboxidivorans*, which was stimulated to convert acids to alcohols by medium acidification when growing on a syngas mixture; however, this phenomenon was not so efficient when growing on glucose (Fernández-Naveira et al., 2017a). CO was also preferred by another acetogen, *C. ljungdahlii*, as a substrate and electron donor for the formation of reduced products such as ethanol since it provides excess NADH for the production of alcohols (Hermann et al., 2020). The summarized results of autotrophic and heterotrophic growth and production of metabolites are provided in Table 1.

4. Conclusions

The influence of the pH value and its regulation on the nature of metabolites produced in *C. aceticum* was shown to depend on the carbon source. When fructose was used as sole substrate, the process suffered from formic acid accumulation, as shown in Table 1, and inhibition was observed as a consequence of reducing equivalents deficiency. A possible suggestion to overcome this problem may be H_2 enhanced mixotrophy in which H_2 is provided to the fermentation medium exogenously. Under the examined conditions, CO can be considered as better carbon source than fructose for the production of ethanol by *C. aceticum*.

CRediT authorship contribution statement

Kübra Arslan: Investigation, Data curation, Writing – original draft. **María C. Veiga:** Data curation, Validation, Supervision, Writing – review & editing. **Christian Kennes:** Conceptualization, Data curation, Validation, Funding acquisition, Supervision, Writing – review & editing.

Declaration of Competing Interest

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

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