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(54) Title: METHOD FOR PRODUCING BIOCHEMICALS FROM CO_2 FERMENTATION

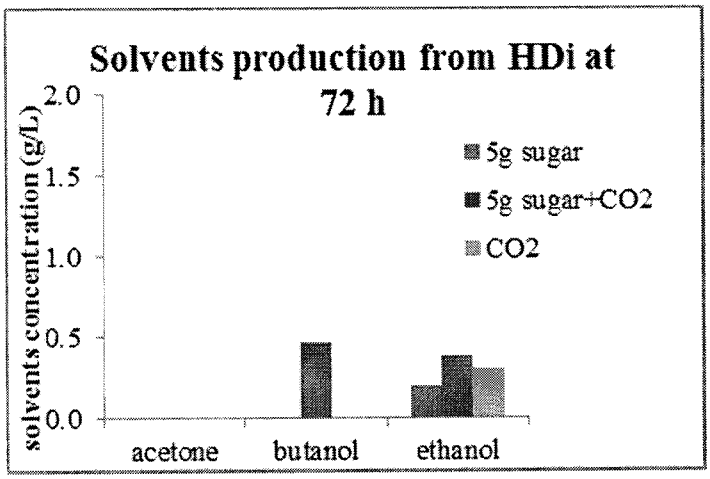

Figure 2

(57) Abstract: The invention provides a method for direct production of biochemicals such as butanol, ethanol and butyric acid from CO_2 as primary carbon source via fermentation without photosynthesis. The microorganisms used for this fermentation process are *Clostridium CGI* and *Clostridium HDi*. The culture medium for this method has been modified to be suitable for growing *Clostridium CGI* and *Clostridium HDi*. In fermentation step, small amount of carbohydrates may be added to enhance productivity rate.

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— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

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METHOD FOR PRODUCING BIOCHEMICALS FROM CO_2 FERMENTATION

TECHNICAL FIELD OF THE INVENTION

This invention relates to a method for producing bioalcohols and organic acids such as butanol, ethanol and butyric acid using carbon dioxide as the main carbon source.

BACKGROUND OF THE INVENTION

There are several ways in producing chemicals from renewable resources such as biomass. One option is by chemical process such as Fischer-Tropsch process, which chemically converts a mixture of carbon monoxide and hydrogen (syn. gas) into target chemicals. Syn. gas can be generated from biomass via gasification; however, Fischer-Tropsch normally requires specific ratio of carbon to hydrogen and limited impurities. In some cases, hydrogen must be added to the mixture. Addition and/or handling of hydrogen requires high standard of safety, which will further drive the cost upward. In addition, biomass gasification and syn. gas clean-up step are not low cost processes.

Another potential process to produce chemicals from renewable resource is through fermentation. Producing biochemical through fermentation is often slower than chemical process; however, fermentation can reduce several complex reactions into one single reactor. Furthermore, fermentation step is often carried out at low temperature and pressure. Nevertheless, fermentation normally uses sugar as raw material, which may lead to food vs. fuel dilemma. In addition, low crude oil price will highly reduce the cost attractiveness of 1st (sugar and starch) and 2nd Generation (lignocellulosic) technologies. There is a need to reduce the use of raw material in fermentation process.

SUMMARY OF THE INVENTION

The present invention discloses a method for producing biochemicals from CO_2 fermentation comprising the steps of: preparing a culture medium, adding CO_2 and sugar to the culture medium, and adding *Clostridum* to the culture medium.

In an embodiment, the method further comprises adding N_2 to the culture medium. The N_2 ranges from 60 to 90% of total combined CO_2 and N_2. To shorten the experiment

time, glacial acetic acid is added to the culture medium in range 1-30 mM to the medium at specific interval time 24, 48, 72, and 96 hour.

In another embodiment of the invention, the bacteria *Clostridium* are chosen from *Clostridium* CG1, *Clostridium* HDi, *Clostridium glycolicum, Clostridium magnum, Clostridium mayombei, Clostridium aceticum, Clostridium formicoaceticum*

In an embodiment, CO_2 fermentation takes place at a temperature from 30 to 60°C for 24 to 120 hours.

In yet another embodiment, CO_2 is added at a flow rate from 0.5 to 5.0 L/minutes, for 15 to 45 minutes. The CO_2 ranges from 10 to 40% of total combined CO_2 and N_2 gas.

In an embodiment, N_2 ranges from 60 to 90% of total combined CO_2 and N_2.

In another embodiment, the method comprises adding a sugar to the culture medium chosen from arabinose, fructose, glucose, mannose, xylose, lactose or cellobiose.

In another embodiment, the method comprises sugar in the range from 10 to 30 g/L.

In another embodiment, the method comprises cellobiose in the range from 0.5 to 10 g/L.

In an embodiment, the culture medium comprises magnesium sulfate ranging from 0.1 to 1.00 g/L, dipotassium hydrogen phosphate ranging from 0.12 to 1.00 g/L, potassium hydrogen phosphate ranging from 0.12 to 1.00 g/L, ferrous sulfate ranging from 0.005 to 0.05 g/L, Resasurin ranging from 0.5 to 5.0 ml, and acetic acid ranging from 1 to 10.0 ml.

In yet another embodiment, the culture medium comprises NH_4Cl ranging from 0.14 to 1.00 g, NaCl ranging from 0.14 to 1.00 g, $MgCl{\cdot}6H_2O$ ranging from 0.1 to 1.00 g, KH_2PO_4 ranging from 0.5 to 2.0 g, K_2HPO_4 ranging from 1.0 to 3.0 g, Trypticase ranging from 1.0 to 5.0 g, yeast extract ranging from 0.5 to 3.0 g, Trace element solution SL-10 ranging from 0.5 to 3.0 ml, $FeCl_3{\cdot}6H_2O$ ranging from 1.0 to 5.0 mg, L-cysteine-HCl ranging from 0.25 to 3.0 g and Resazurin ranging from 0.2 to 2.0 mg.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 illustrates a flow chart of the method for producing biochemicals from CO_2 fermentation

Figure 2 illustrates a comparison of solvent production from three sources of carbon using *Clostridium* HDi

Figure 3 illustrates a comparison of solvent production from three sources of carbon using *Clostridium* CG1

Figure 4 illustrates a graph of an experiment comparing the conventional technology with hybrid technology and CO_2 technology using *Clostridium* CG1

Figure 5 illustrates a graph of an experiment comparing the conventional technology with hybrid technology and CO_2 technology using *Clostridium* HDi

DETAILED DESCRIPTION OF THE INVENTION

The present invention reduces the issue of raw material via direct conversion of CO_2 into chemicals via fermentation instead of using renewable resources such as sugarcane or biomass generated from CO_2 via photosynthesis. CO_2 is usually very unreactive, but easy to find as by-product of large industries (e.g. exhaust of power plant). By eliminating photosynthesis step, the dark fermentation is possible, which will eliminate indirect competition with food (land) when compared to 3rd gen. solution like microalgae and save a huge amount of energy via lack of extraction step. Beside the reduction of resources needed, the biocatalysts use in this invention have a faster growth rate than microalgae, and able to generate products at higher concentration, thus generate further cost saving in the downstream purification steps.

The present invention provides a method for producing biochemicals via CO_2 fermentation. This invention is different from syn. gas fermentation process due to the lack of costly gasification and syn. gas upgrading (hydrogen gas addition), and CO2 as primary carbon source instead of CO. Without the use of hydrogen, the safety standard and the cost related with it can be significantly reduced given CO_2 is an inert gas. Furthermore, this

process can be linked to power plant that produces CO_2 as a by-product of combustion or other processes that produce CO_2 as by-product. This process linkage is not possible with traditional syn. gas fermentation process.

The *Clostridium* to be used in this fermentation process is isolated to identify the *Clostridium* that can use CO_2 as a substrate. Two strains of *Clostridium* that are able to use CO_2 as a substrate without using any other carbon source have been identified and isolated. They are *Clostridium* CG1 and *Clostridium* HDi. These two strains have been used to produce biochemicals using fermentation under the condition where only CO_2 is used as a carbon source. These two strains can also produce biochemicals using fermentation under the condition where CO_2 and sugar are used as carbon sources.

The method for producing biochemicals from CO_2 fermentation is shown in Figure 1. The method for producing biochemicals from CO_2 fermentation comprises preparing a culture medium, adding CO_2 and sugar to the culture medium, and adding *Clostridium* to the culture medium. To shorten the experiment time, glacial acetic acid is added to the culture medium in range 1-30 mM to the medium at specific interval time 24, 48, 72, and 96 hour. Fermentation takes place at a temperature from 30 to 60°C for 24 to 120 hours.

In an embodiment, the culture medium is a modified synthetic medium which has been altered suitable for growing *Clostridium* CG1. The culture medium comprises magnesium sulfate ranging from 0.1 to 1.00 g/L, dipotassium hydrogen phosphate ranging from 0.12 to 1.00 g/L, potassium hydrogen phosphate ranging from 0.12 to 1.00 g/L, ferrous sulfate ranging from 0.005 to 0.05 g/L, Resasurin ranging from 0.5 to 5 ml and acetic acid ranging from 1 to 10 ml. The pH is adjusted to 4 to 8 using ammonium. The culture medium is put to a boil for 1 to 10 minutes. CO_2 is then added as a substrate to the culture medium for 15 to 45 minutes at the gas flow rate of 0.5 to 5 L/min. The CO_2 is added to displace oxygen. In this process it is possible to use only CO_2 gas as substrate. Thus, up to a 100% CO_2 can be added without using any other gas for the fermentation process.

In another embodiment, nitrogen is added to the culture medium in addition to the CO_2. The amount of nitrogen added ranges from 60 to 90 %. Alternatively, nitrogen ranges

from 60 to 80%. When nitrogen is added, the amount of CO_2 ranges from 10 to 40% or 20 to 40%. The CO_2 and nitrogen substrate is added for 15 to 45 minutes.

In yet another embodiment, sugar can be added to the culture medium. The sugar can be arabinose, fructose, glucose, mannose, xylose, lactose or cellobiose. The sugar can range from 10 to 30 g/L. The cellobiose can range from 0.5 to 1 g/L.

In an embodiment, the culture medium is a Caldicellulosiruptor medium which has been altered suitable for growing *Clostridium* HDi. The culture medium comprises NH_4Cl ranging from 0.14 to 1.00 g, NaCl ranging from 0.14 to 1.00 g, $MgCl{\cdot}6H_2O$ ranging from 0.1 to 1.00 g, KH_2PO_4 ranging from 0.5 to 2.0 g, K_2HPO_4 ranging from 1.0 to 3.0 g, Trypticase ranging from 1.0 to 5.0 g, yeast extract ranging from 0.5 to 3.0 g, Trace element solution SL-10 ranging from 0.5 to 3.0 ml, $FeCl_3{\cdot}6H_2O$ ranging from 1.0 to 5.0 mg, L-cysteine-HCl ranging from 0.25 to 3.0 g, and Resazurin ranging from 0.2 to 2.0 mg. The pH is adjusted to 7.2 using ammonium. The culture medium is put to a boil for 2 to 5 minutes. 20% CO_2 and 80% N_2 is then added as a substrate to the culture medium for 15 minutes. The culture medium is autoclaved for 15 minutes.

Clostridium is a genus that includes many species. In general, CO_2 utilized *Clostridium* has been reported in the species of *Clostridium autoethanogenum, Clostridium ljungdahlii and Clostridium carboxidivorans* in previous reports. *Clostridium CG1, Clostridium HDi, Clostridium glycolicum, Clostridium magnum, Clostridium mayombei, Clostridium aceticum, Clostridium formicoaceticum* have now been found to use CO_2. *Clostridium* CG1 and *Clostridium* HDi have been selected for the following examples.

Example 1:

Clostridium CG1 and *Clostridium* HDi are fermented three ways using sugar, sugar + CO_2 or CO_2 as carbon sources. After 72 hours of fermentation the broth is analyzed using gas chromatography. The column used is DB-WAX, Shimadzu column, size 30 X 0.53 m. The carrier gas is helium 50 to 100 ml/min. Flame ionization detector is used at 220 to 240°C. The temperature of the column oven is 45 to 55°C. The result is compared to standard graph for analyzing the concentration of butanol and ethanol. Figures 2 and 3 show a comparison of solvent production from the different types of fermentation using sugar, sugar + CO_2 and CO_2

as carbon sources. Figure 2 shows the result for *Clostridium* HDi and Figure 3 shows the result for *Clostridium* CG1. It is apparent from these Figures that the optimal condition is sugar and CO_2. It should be noted that ethanol is produced in fermentation using only CO_2 as carbon source in both *Clostridium* HDi and *Clostridium* CG1.

Example 2:

Figure 4 and 5 show graphs of experiments comparing the conventional technology with hybrid technology and CO_2 technology. The conventional technology is fermentation using *Clostridium* and sugar as a carbon source. The hybrid technology is fermentation using *Clostridium,* sugar and CO_2 as carbon sources. The CO_2 technology is fermentation using *Clostridium* and CO_2 as a carbon source. *Clostridium* CG1 is used in the experiment shown in Figure 4 and *Clostridium* HDi is used in the experiment shown in Figure 5.

In Figure 4, when sugar and CO_2 are used as carbon sources *Clostridium* CG1 is able to produce butanol 1.4 times higher and ethanol 2 times higher than the conventional technology. In Figure 5, when sugar and CO_2 are used as carbon sources *Clostridium* HDi is able to produce butanol whereas butanol is not produced using the conventional technology. In the hybrid technology ethanol is produced 1.8 times more than the conventional technology. Furthermore, when using the CO_2 technology, *Clostridium* HDi is able to produce more butyric acid than the hybrid technology.

CLAIMS

1. A method for producing biochemicals from CO_2 fermentation comprising the steps of:

preparing a culture medium;

adding CO_2 and sugar to the culture medium; and

adding *Clostridium* to the culture medium.

2. The method of claim 1 further comprises adding N_2 to the culture medium.

3. The method of claim 1 further comprises adding 1 – 30 mM glacial acetic acid.

4. The method of claim 3, wherein the glacial acetic acid is added at interval time 24, 48, 72, and 96 hour.

5. The method of claim 1, wherein the clostridium is chosen from *Clostridium* CG1, *Clostridium* HDi, *Clostridium glycolicum, Clostridium magnum, Clostridium mayombei, Clostridium aceticum, or Clostridium formicoaceticum.*

6. The method of claim 1, wherein fermentation takes place at a temperature from 30 to 60°C for 24 to 120 hours.

7. The method of claim 1, wherein CO_2 is added at a flow rate from 0.5 to 5.0 L/minutes.

8. The method of claim 1, wherein CO_2 is added for 15 to 45 minutes.

9. The method of claim 1, wherein CO_2 ranges from 10 to 40% of total combined CO_2 and N_2 gas.

10. The method of claim 1, wherein N_2 ranges from 60 to 90% of total combined CO_2 and N_2.

11. The method of claim 1 further comprises adding a sugar to the culture medium chosen from arabinose, fructose, glucose, mannose, xylose, lactose or cellobiose.

12. The method of claim 9, wherein the sugar ranges from 10 to 30 g/L.

13. The method of claim 9, wherein the cellobiose ranges from 0.5 to 10g/L.

14. The method of claim 1, wherein the culture medium comprises:

magnesium sulfate ranging from 0.1 to 1.00 g/L;

dipotassium hydrogen phosphate ranging from 0.12 to 1.00 g/L;

potassium hydrogen phosphate ranging from 0.12 to 1.00 g/L;

ferrous sulfate ranging from 0.005 to 0.05 g/L;

Resasurin ranging from 0.5 to 5.0ml; and

acetic acid ranging from 1 to 10.0 ml.

15. The method of claim 1, wherein the culture medium comprises:

NH_4Cl ranging from 0.14 to 1.00 g;

NaCl ranging from 0.14 to 1.00 g;

$MgCl \cdot 6H_2O$ ranging from 0.1 to 1.00 g;

KH_2PO_4 ranging from 0.5 to 2.00 g;

K_2HPO_4 ranging from 1.0 to 3.0 g;

Trypticase ranging from 1.0 to 5.0 g;

yeast extract ranging from 0.5 to 3.0 g;

Trace element solution SL-10 ranging from 0.5 to 3.0 ml;

$FeCl_3 \cdot 6H_2O$ ranging from 1.0 to 5.0 mg;

L-cysteine-HCl ranging from 0.25 to 3.0 g; and

Resazurin ranging from 0.2 to 2.0 mg.

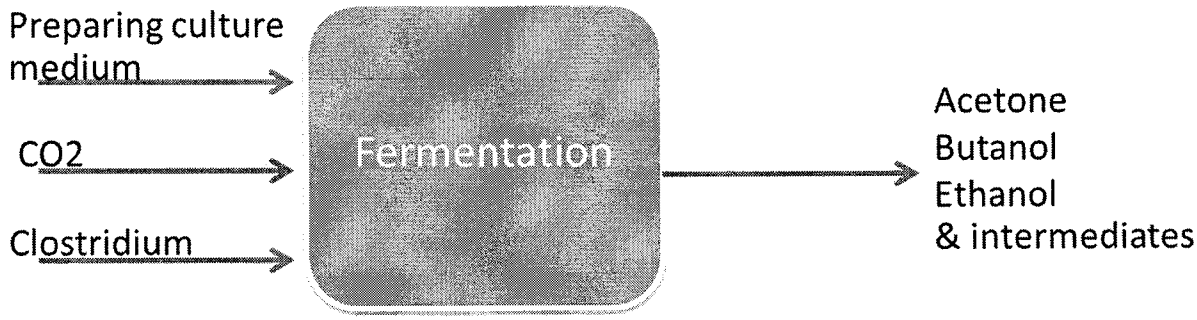

Figure 1

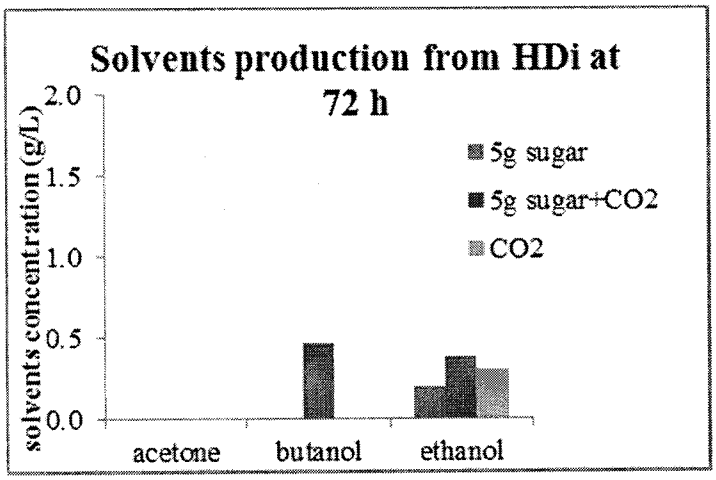

Figure 2

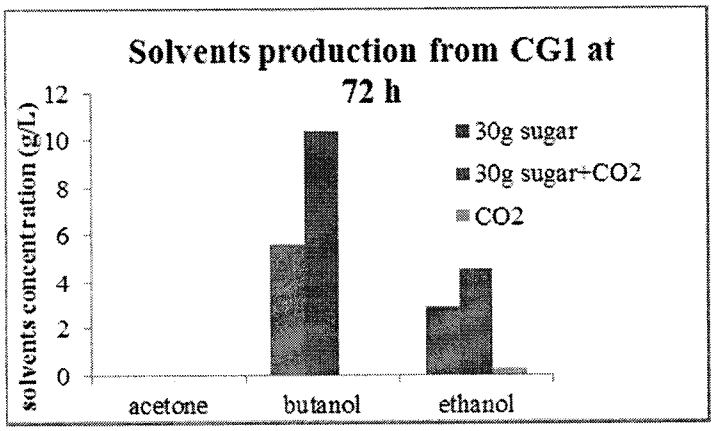

Figure 3

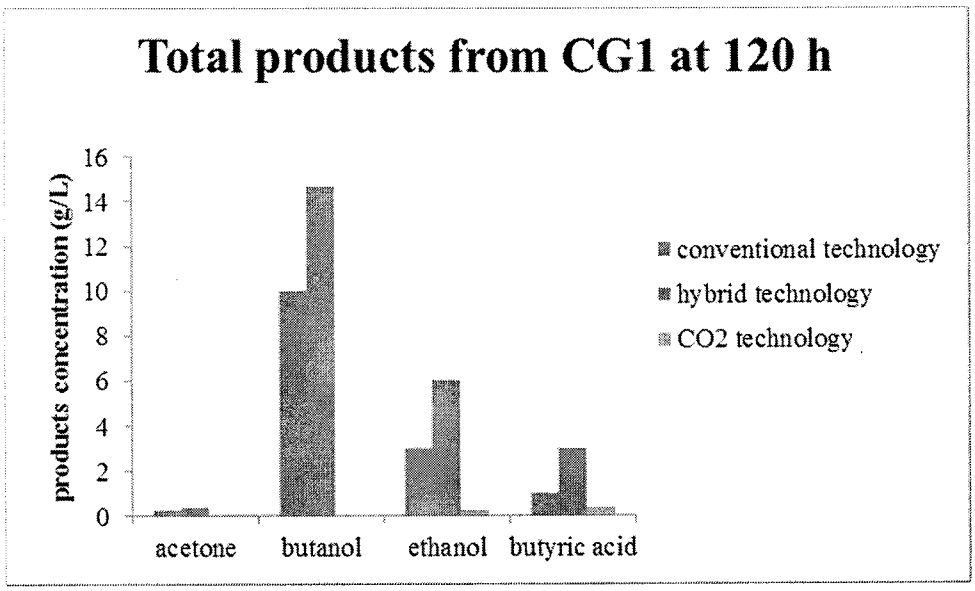

Figure 4

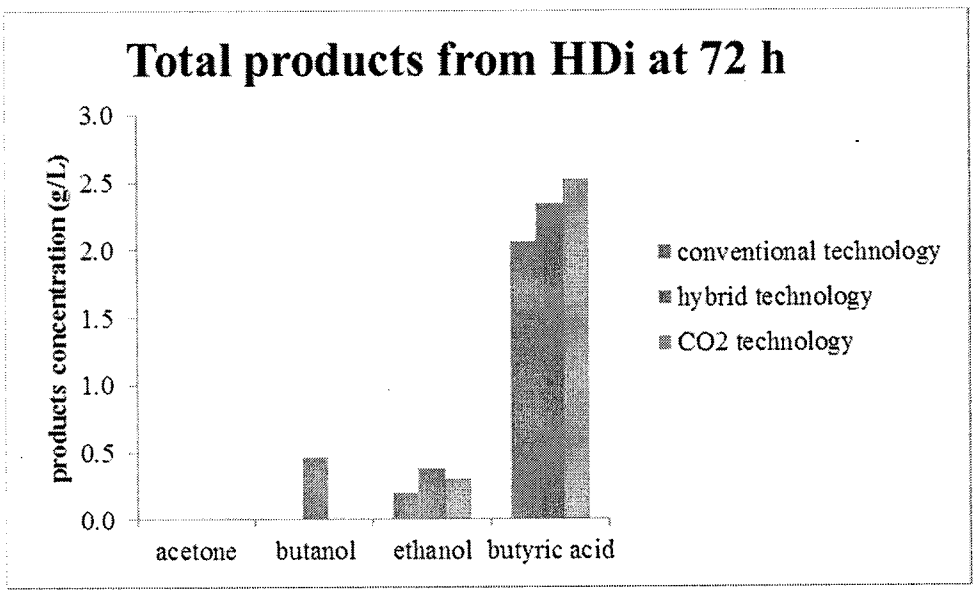

Figure 5

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER

Int.Cl. C12P7/16(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int.Cl. C12P7/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Published examined utility model applications of Japan 1922-1996
Published unexamined utility model applications of Japan 1971-2016
Registered utility model specifications of Japan 1996-2016
Published registered utility model applications of Japan 1994-2016

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

JSTPlus/JMEDPlus/JST580 (JDreamIII), CAplus/MEDLINE/EMBASE/BIOSIS/WPIDS (STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X Y	JP 6-165685 A (KUBOTA CORPORATION) 1994.06.14, Claims, Examples, [0005] (family : none)	1,6,11 1-15

☑ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
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Date of the actual completion of the international search	Date of mailing of the international search report
15.08.2016	30.08.2016

Name and mailing address of the ISA/JP	Authorized officer	
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/TH2016/000021

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	COMWIEN, J. et al., Direct Production of Butanol and Ethanol from Cane Sugar Factory Wastewater and Cellulosic Ethanol Pilot Plant Wastewater by *Clostridium Beijerinckii* CG1, Energy Procedia, 2015, Vol.79, pp.556-561, Abstract, Materials and Methodology, Fig.1	1-15
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/TH2016/000021

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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