Report Regarding January-November 2016 Research Phase

1. **Obtaining lactic acid and other biochemicals from sorghum juice**

The project team carried out research regarding cultivation of lactic acid bacteria using as fermentation broth sorghum juice to produce lactic acid. The lactic acid bacteria strains tested for the production of lactic acid are originating from the Collection of Microorganisms of Industrial USAMVB Timişoara (CMIT) and are the followings: *Lactococcus lactis CMGB 31*, *Lactobacillus sp.* CMIT 1.45, *Lactobacillus pentosus* DSM 20314, *Lactobacillus acidophilus* CMIT 1.49, *Lactobacillus sp* CMIT 1.47, *Lactobacillus sp* CMIT 1.48, *Lactobacillus plantarum* CMIT 1.46, *Enterococcus faecium CMIT 1.57*, *Lactobacillus paracasei* CMIT 1.55, *Lactobacillus helveticus* CMIT 1.51, *Lactobacillus rhamnosus CMIT 1.51*, *Streptococcus faecalis* CMIT 1.61, *Lactobacillus sporogenes* (*Bacillus coagulans*) CMIT 1.62. In the first stage bacteria have been cultivated in specific media (MRS and GSY) modified to obtain a sugar composition similar to that found in sorghum juice (glucose, sucrose and fructose). The fermentation was carried out under static conditions at 37°C, 48 hours. Samples were taken at 0, 4, 8, 16, 24, 28, 36, 48 hours. Samples were analyzed regarding the concentration of reducing sugars, glucose, sucrose, D and L forms of lactic acid and acetic acid. Our results made possible the assessment of the production capacity of lactic acid from this type of materials. Thus, we have obtained lactic acid productivities between 70 and 85% based on consumed sugars. Depending on the process applied for extracting sugars from sorghum biomass, carbohydrate solutions were obtained with total sugar concentrations between 14 and 24%, which allows obtaining lactic acid productions of 11-18 g / 100 ml of juice sorghum. Reported at the total amount of sorghum strains, the production potential of lactic acid can reach 550 kg / ton DM biomass sorghum stalks. Lactic acid (isomers D and L) is used for the production of PLA, both types of isomers are applied in the production of biodegradable plastics. Calcium lactate is a product used in large amounts in human and veterinary medicine. Another approach is the anaerobic fermentation of the sugars obtained by hydrolysis of the cellulose from sorghum bagasse by Clostridium species for the production of second generation acetone, butanol and ethanol. The first preliminary results obtained by the project team regarding extraction of sugars from sorghum stalks, hydrolysis of cellulose from waste and fermentation of these sugars were sent for publication: Teodor Vintila, Vasile Daniel Gherman, Nicolae Popa, Dumitru Popescu, Adina Negrea, Influence of

Enzymatic Cocktails on Conversion of Agricultural Lignocellulose to Fermentable Sugars, Revista de Chimie, (I.F. 0,81) and presented in internaționale conferences: Teodor Vintilă, Nicolae Popa, Adrian Trulea, Iosif Gergen, Kornel Kovacs, Biorefinery of sweet sorghum to produce biogas and other biochemicals, Biogas Science 2016, 21-24 August 2016, Szeged, Hungary; Mild alkaline pretreatment applied in the biorefinery of sorghum biomass for ethanol and biogas production, Agriculture for Life, Life for Agriculture, 9-11.06.2016, București, România; Sugar Production Potentials of Some Sweet Sorghum Hybrids Cultivated in Heavy Metals Polluted Soil, International Conference for Environmental and Agricultural Engineering, August 14-16, Porto, Portugalia; Biorefinery of biomass to obtain biofuels and bioplastics, conference Bioenergy in Romania, in RoEnergy Expo-Fair, 20.10.2016, Timișoara, Biorefinery of sweet sorghum for a circular economy, First European Sorghum Congress, 3-4 November 2016, București, Romania.

2. **Improving energetic balance through cascade processing of sorghum to obtain biogas**

In this work package the project team approached the biorefinery process by connecting in cascade previously proved biotechnologies using sorghum biomass as feedstock. In order to maximize the energy output of the raw material processed, three different conversion pathways were examined:

1. Untreated biomass to biogas via anaerobic digestion;
2. Pretreated biomass to biogas via anaerobic digestion;
3. Pretreated biomass to ethanol via alcoholic fermentation followed by anaerobic digestion of the spent fermentation mass resulted after the alcoholic fermentation.

Biomass from three different sorghum hybrids, namely *Sorghum bicolor x sudaneze* cv. Jumbo, originally from Australia, *Sorghum bicolor x sudaneze* Sugargraze II, originally from the United States of America and *Sorghum bicolor var. Saccharatum* cv. F135ST, originally from Romania, was used as biological raw material. The biomass was air – dried after harvest and then milled using the Retsch SM100 mill purchased with founds provided by this grant.

After milling, the biomass was subjected to pretreatment process. The next step of the process consisted of enzymatic hydrolysis of pretreated biomass. Fermentation was carried out with *Saccharomyces cerevisiae* in 500 ml flasks equipped with ethanol and CO_2 sensors from Blue Sens - Germany . Anaerobic digestion of the biomass was carried out according to the VDI 4630 guidelines (VDI, 2006) in batch tests using the AMPTS II system containing 15 fermenters (Bioprocess Control – Sweden) purchased with founds provided by this grant. The obtained results show important differences in biogas and methane yields obtained in the case of anaerobic digestion of untreated and pretreated biomass. Regarding the methane yield, the pretreatment increases productions with an average of 61 l_N/kg organic dry matter, which is translated as 21% increase of methane yields. In the batches performing anaerobic digestion of the spent fermentation mass resulted after alcoholic fermentation of the pretreated sorghum biomass higher yields were obtained. In all three sorghum varieties, the methane yields were in average 49% higher than in the case of the untreated batches and 22% higher than in the case of the of the pretreated batches. Although parts of the carbohydrates present in the biomass were degraded and converted into ethanol during the fermentation process, the highest yields of biogas and methane were recorded in the samples containing spent fermented mass. This atypical result can have two reasons at its foundation.

First of all, during the alcoholic fermentation of the biomass, yeasts convert the soluble sugar faction not only into ethanol, which is extracted by distillation. During the growth process, yeasts synthesize proteins and other biomolecules which are used as substrate by the microorganisms employed in the next phase of anaerobic digestion process and converted into biogas.

Secondly, the alcoholic fermentation process can be considered as an additional biological pretreatment step that loosens the bonds in the lignocellulosic structure and thus liberating an extra amount of carbohydrates that can be converted in the anaerobic digestion step. Between the three different conversion pathways studied in this work package: (1) Anaerobic digestion of untreated biomass; (2) Anaerobic digestion of pretreated biomass; (3) Anaerobic digestion of bagasse resulted after alcoholic fermentation of pretreated biomass; the highest yields were obtained in the third conversion pathway, by connecting ethanol fermentation to anaerobic digestion. This means that an integrated conversion pathway constructed by connecting in cascade the second generation bioethanol production with anaerobic digestion is the most suitable in terms of energy yields from biomaterials such as sorghum biomass.

An important aspect of our research in this project was to obtain sorghum field trials on soils polluted with heavy metals in Copsa-Mica area, Sibiu County. The purpose of this research is not only phytoremediation of polluted soils, but also exploiting the potential of polluted areas, where is not recommended to produce agricultural products for use in food or feed purposes. Moreover, we followed the distribution of heavy metals in various fractions, products and by-products generated during biorefineriy of heavy metals contaminated biomass. Sorghum

obtained on these soils can be used exclusively for non-food industrial applications such as the production of renewable energy, according to the technology described in this study.
The pretreated biomass was hydrolyzed using cellulases and fermented to ethanol with *Saccharomyces cerevisiae*. The concentration of the metals in the solid phase of the hydrolysis/fermentation broth has increased, due to solubilisation of the main fraction of the organic solids, and a fraction of the metals (especially Zn was found in the liquid phase of the fermentation medium). Sorghum crops obtained on polluted soil can be used as substrate for lignocellulosic ethanol production. The main part of the polluting metals remained in the solid residue, a small fraction in the distillation residue, and traces of Pb and Cu could be found in the distilled ethanol. Residues were subjected to anaerobic digestion to obtain biogas and the digestate, which can be returned to the same polluted fields as fertilizer.
Scaling up this process to industrial level can be a useful solution to utilize such soils for production of renewable energy instead using them for producing food or feed containing heavy metals. The conversion of biomass to energy carriers as ethanol and methane can be conducted in a biorefinery placed inside the polluted/contaminated area, and therefore, the transportation costs will be reduced. Importantly, the residue (the digestate) containing the polluting metals can be distributed to the same polluted fields, thus preventing pollutant spreading to other areas, such as in the case of scattering of ash reach in heavy metals resulted from burning biomass produce in polluted areas.
The results obtained in this research have been published or are in press in: Adrian Trulea, Teodor Vintilă, Nicolae Popa, Georgeta Pop, Mild alkaline pretreatment applied in the biorefinery of sorghum biomass for ethanol and biogas production, AgroLife Scientific Journal accepted in Issue 2, December 2016 (journal indexed Thomson Reuters); Teodor Vintilă, Nicolae Popa, Adrian Trulea, Iosif Gergen, Kornel Kovacs, Biorefinery of sweet sorghum to produce biogas and other biochemicals, Biogas Science 2016, 21-24 August 2016, Szeged, Hungary; Teodor Vintila, Adina Negrea, Horia Barbu, Radu Sumalan, Kornel Kovacs, Metal Distribution in the Process of Lignocellulosic Ethanol Production from Heavy Metal Contaminated Sorghum Biomass, Journal of Chemical Technology & Biotechnology Volume 91, Issue: 6, 2016, 1607-1614, IF: 2,349; Teodor Vintilă, Adrian N. Trulea, Nicolae Popa, Daniela Vintila, Georgeta Pop, Sugar Production Potentials of Some Sweet Sorghum Hybrids Cultivated in Heavy Metals Polluted Soil, Journal of Advanced Agricultural Technologies, ISSN: 2301-3737 (online) accepted in volume V4N1 MAR. 2017.

1. Optimization of entire process

In this activity, we have approached an integrated biorefinery concept of sugar plants, approaching sweet sorghum as a case study. Biorefinery applied in this project is the preservation of sugars by aditivate ensilage of freshly harvested sorghum biomass, extracting sugars from biomass silage, followed by fermenting sugars with the production of ethanol, hydrolysis without pretreatment of lignocellulose, obtaining ethanol from cellulosic hydrolysate and anaerobic digestion of residues from the fermentation and hydrolysis and production of biogas and organic fertilizer.

As a result of the studies conducted in this phase of the project we have generated an original biorefining process, subject to patenting, (*Patent Application no. 00334 A / 11.05.2016, Title of the invention: Process for biorefining of sugar plants with preservation and extraction of sugars for the production of biofuels and other biochemicals. Owner: USAMVB Timisoara. The patent application will be published in: BOPI 11/2016, November 2016*).

The original biorefining process of sugar plant developed in this project provides the following advantages:

- The additive used as conservation and extracting agent is recovered from silage material and can be reused for the following extractions;

- During storage and extraction of the sugars from the biomass of sugar plants, te solution added as preservative has the effect of pretreatment of lignocellulosic biomass so that it is not necessary a further steps of pretreatment of lignocellulosic complex prior to enzymatic hydrolysis of cellulose to obtain fermentable sugars.

- The biogas yields obtained by anaerobic digestion of generated in alcoholic fermentation of sweet sorghum is higher than the amount of biogas produced from whole biomass of sweet sorghum that has not been used in alcoholic fermentation.

- By chaining the three main processes: (1) alcoholic fermentation of sugars extracted from sweet sorghum (2) enzymatic hydrolysis and fermentation of bagasse results after extraction of sugars and (3) anaerobic digestion of waste resulted after hydrolysis and fermentation for biogas production, total energy production is maximized, and at the end of the process it is obtain a valuable organic fertilizer.

After 6 months of storage under the conditions described, sugar concentration in the liquid harvested from silos increased up to 150 mg / ml. Throughout the 12-month period of storage and extraction of the sugars, the concentration of sugars in the juice maintained approximately constant and at the last harvest after 12 months of storage, liquid with concentration of 136 mg / ml was obtained. Regarding the amount of juice harvested, this may vary depending on the type of press used and the pressing force of biomass. Another aspect relating to the extraction of the juice, it can be removed by leaching, without pressing. By these repeated extractions, sugars output relative to the amount of extracted sorghum biomass is increased comparing to the process with single extraction.

By hydrolysis and fermentation of bagasse resulted after repeated flooding extraction process 1000 liters / hectare of ethanol was obtain. This output is higher than the production of ethanol obtained by high-pressure / alkaline steam pretreatment and enzymatic hydrolysis of the fermented bagasse resulted after solid state fermentation of sorghum biomass.

These results brings an extremely important novelty, namely: treating for several months lignocellulosic biomass with preservative solution has better effect in terms of improving access of enzymes for hydrolysis of cellulose than conventional methods of physicochemical pretreatment (alkaline / steam). This has a major impact in the economy of biorefining processes of plant biomass, because the thermos-chemical pretreatment steps are energy intensive and polluting. In other words, treatment of sugary plant biomass according to patenting process has triple effect: (1) preservation of sugars, (2) extraction of sugars and (3) release of cellulose from lignocellulosic complex and access of cellulolytic enzymes to hydrolysis cellulose to glucose.

Innovative biotechnology developed by the project team enables processing sugar plant biomass (especially sorghum) all year round, not just shortly after harvest (because sugars are lost in time). More than that, ethanol is obtained from extracted sugars, second generation ethanol or other biochemicals from cellulose and biogas from fermentation and hydrolysis residues without applying physical or chemical methods of pretreatment of lignocellulosic biomass.

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