Newsletter 05

June 2014

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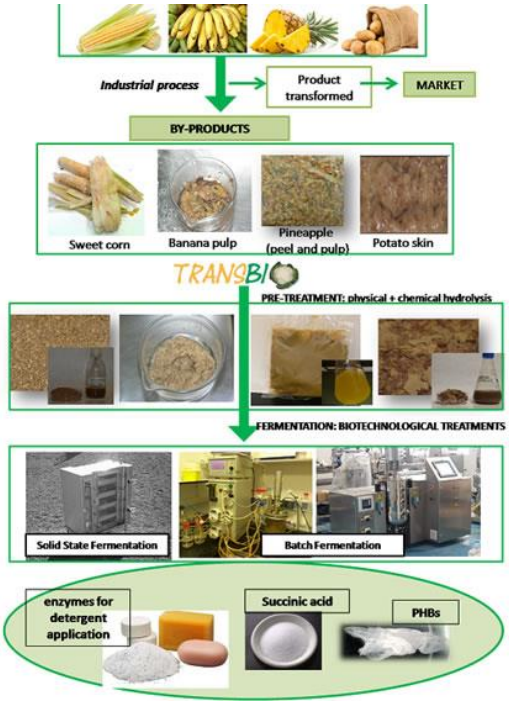
TRANSBIO in a nutshell...

The European research project TRANSBIO aims in the implementation of an innovative cascading concept for the betterment of sub-products using environmental friendly biotechnological solutions like fermentation and enzyme-conversion strategies to obtain valuable bio-products like biopolymers (PHB), nutraceuticals / platform chemical (succinic acid) and enzymes for detergent applications.

Thus the overall sustainability of biomass processing industry will be improved and the competitiveness of European biotechnology industry will be increased through new applications.

In order to target and realize different biotechnological approaches for transforming by-products from fruit and vegetable processing industry into value-added bioproducts, the consortium consist of 16 partners coming from nine countries and two continents (Europe / Latin America) combining their knowledge & experiences to reduce production costs for biopolymer PHB, bio-based succinic acid and enzymes for detergent application.
Finally, remaining biomass will be evaluated for their potential to be used for biogas production.

In order to achieve the TRANSBIO strategy, the project has been designed as a cascade of technical work packages (WP1-12) with continuous progress assessment between the individual scientific and methodological approaches. In parallel, economic and environmental evaluation (WP14), testing activities in pilot plant scale (WP13), as well as dissemination activities (WP15) will be performed.



Work progress

TRANSBIO started its research activities with definition of requirements in WP1. Beside state-of-the art analysis, sustainability criteria were established. In order to analyse the economic performance of the new processes an Excel Spread sheet was established to include continuously process data. Additional value-added chains of the new processes/products were investigated as well as legal- and end-user requirements.

WP2 started its work with the selection of possible by-products to be used. Based on literature and company information a first selection was made and the partners agreed about analytical methods. Afterwards a timetable with the availability of the different sub-products was prepared and samples were sent around for analysis. Additional, experiments related to drying and physical disintegration took place as well as chemical and enzymatic hydrolysis. First hydrolysates were analysed and sent to fermentation partners.

In **WP3** molecular screening procedures for the selection of PHB producing candidate strains were established. 200 possible PHB producing bacteria could be identified. Additional a first prototype of a recombinant E. coli was designed.

In **WP4** the PHBs production has been tested with two fermentation substrates obtained from the hydrolysis processes of two of the by-products proposed in this project: potato skins and sweet corn wastes.

Following table shows the composition of the fermentation substrates:

component (g/L)	potato hydrolysate (formulation for fermentation: 25 %w/v)	sweet corn hydrolysate (formulation for fermentation: 10 %w/v)
glucose	11,2	25,9
fructose	< 1	7
maltose	2,5	< 1
sucrose	< 1	< 1
Total Reducing sugars	**21,1**	**35,0**
nitrogen	1,1	0,9

PHBs production has been optimized in BATCH fermentation at volumes of 2 liters.

The highest PHBs concentration has been obtained with a wild strain codified as M38 and identified as *Cupridavidus necator*. This strain has reached a concentration of 5.8 g/L of PHBs using as fermentation substrate enzymatic hydrolysate of potato skins.

Tests have been performed at lab scale.

Extraction and purification of the introcitoplasmatic granules of PHBs have been developed by the conventional methods and the first samples of plastic polymers have already been obtained.

Next steps in WP4 will try to reduce the use of organic solvents in the extraction and purification stage and will be focused in the improvement of the PHBs yields by scaling the fermentation process up to 30 liters.

In **WP6** the analysis of the microorganisms, particularly yeasts, associated to by-products from fruit and vegetable processing industries was completed by UMinho and our study is the first reporting the highly diverse yeast microbiome in this type of substrates. A total of 701 yeast and 938 bacterial isolates were collected from the fermentations of fruit and vegetable wastes provided by PROMIC. A higher number of yeast isolates was recovered in fruit samples contrary to the vegetable samples, where more bacterial isolates were isolated. No yeast isolates were recovered from broccoli and cauliflower (edible stems). Considering only the yeast isolates, we observed that the number of yeasts recovered from each biowaste was dependent on the type of biowaste and the sample treatment performed. A total of forty-five different yeast species were identified. Candida tropicalis was isolated from nine different biowaste, followed by Pichia fermentans and P. kudriavzevii associated with eight and seven biowaste, respectively. However, the majority of the species, 28 species, were identified in only one type of biowaste. The metagenome analysis of the fruit and vegetable biowaste enabled a comprehensive insight into the microbial diversity within these habitats, as well as a global analysis of the relative abundance of the different microbial species, including non-cultivable microbial species. By metagenomic analysis the predominant genera found were Candida and Saccharomyces, identified in all biowaste. Other genera such as Hanseniaspora, Pichia and Torulaspora were present in 10 biowaste. Analyzing the diversity of yeasts, it is clear that metagenomic approach exhibited greater yeast diversity than culture-dependent methods in all agrofood biowaste. For example, in fresh chard, pear and pepper biowaste, no yeast colonies were recovered before fermentation, while by metagenomic analysis different yeast genera and species were detected. Considering both methods, the most representative genera present in agrofood biowaste were: Candida, Pichia, Saccharomyces, Torulaspora, Hanseniaspora and Rhodotorula.
Characterization of Saccharomyces and non-Saccharomyces yeast strains for succinic acid production using different culture media has been performed and succinic acid production was shown to be strain and culture medium dependent. Some “non-conventional” yeast strains isolated from the biowaste were shown to be good candidates for succinic acid production.
Evaluation of the differences among S. cerevisiae strains regarding succinic acid production in different nutritional conditions has also been studied. The results of these experiments have been used to select not only the strains, but also the fermentation conditions to be applied in the transcriptomic analysis that is presently underway.

In **WP7** the most promising strains identified during WP6 are being challenged against the raw-material hydrolysates produced in WP2 in view of selecting raw-material/strain pairs that result in higher productivities and yields. These trials will be performed at micro scale in micro titre plates with enhanced mass transfer efficiency and in shaken flasks and the concentrations of carbon sources and organic acids measured to gather the required information for proper assessment of the most promising conditions to be implemented at higher scale. Following that, the fermentation process will be optimized and intensified in fully controlled bioreactors for the implementation of a high-productivity process with high reproducibility and robustness. At this scale it will be possible to produce broth samples for the downstream processing activities of WP8. Once the optimized process has been established, it will be translated to successively higher scales, up-to a scale of 200L. These trials will not only contribute

to optimized fermentation strategies at high scale, but will also provide technical data to be included in the feasibility assessment of WP14.

WP9 is dedicated to the filamentous fungi selection and development of lab-scale SSF. Lipases (EC3.1.1.3) are defined as enzymes that break down fat molecules and catalyze the hydrolysis of long triacylglycerol chains to form glycerol and fatty acids in the presence of a water chain. They may also catalyze the reverse reaction, the synthesis of triglycerides under no aqueous conditions

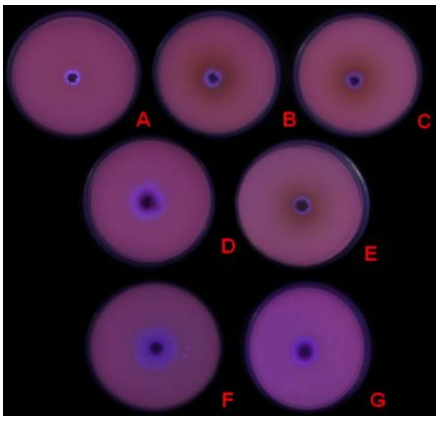
Lipases have a wide range of applications in manufacturing, among the most demanding applications are included its use as additives in different types of detergents (industrial, contact lenses, dishwasher, laundry, etc.). The lipases are usually added to the detergent formulation, especially in combination with proteases, mannanases, peroxidases, amylases etc.

In 1994 Novo Nordisk presented Lipolase, the first fungal commercial enzyme to be used as detergent additive produced by the strain *T. lanuginosus* and expressed in *Aspergillus oryzae*. In 1995, Genencor International produced two bacterial lipases, which were Lumafast™ from *Pseudomonas mendocina* and Lipomax™ from *Pseudomonas alcaligene.*

The methods normally used in the identification of lipolytic microorganisms are solid agar plates which are grouped into two categories: methods based on changes in the appearance of the substrate as a result of lipolysis and the methods that include the use of an indicator dye to detect lipolysis. In both methods, it is important the full contact between the substrate and the enzyme. Therefore, the agar content may be reduced to increase the diffusibility of the extracellular lipase.

For lipases production it is necessary to consider the growing conditions affecting the synthesis of the lipase (the source of carbon and nitrogen, the presence of activators and inhibitors, incubation temperature, pH, amount of inoculum and oxygen tension). The carbon source has been reported as a major factor determining the expression of lipases, and it has been observed that production of these enzymes is dependent on the presence of a lipid compound as a carbon source and olive oil is the most common carbon source used as inductor. In addition, for lipases to be used in detergent formulation it is need also to meet a minimum of requirements in terms of stability to factors such as alkaline pH values, thermostability (30-50 ^{0}C), presence of surfactants, tensoactives, reducing or oxidant agents; water softeners, dyes, brighteners as well to the presence of other enzymes such proteases, mannanases, amylases and finally they must also have broad substrate specificity to be able to hydrolyze fats of various compositions.

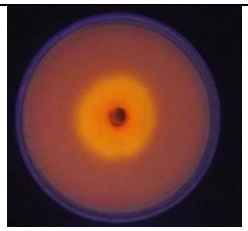
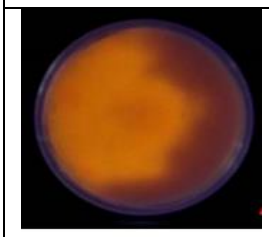
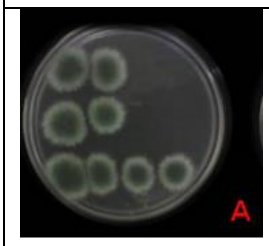
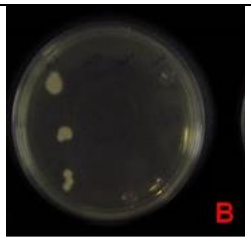
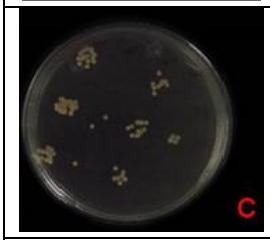
In this sense, the TRANSBIO project has identified the by-products resulted from the extraction of sunflower oil as potential substrate for the isolation of lipolytic microorganisms as well as for the production of lipases for its use in the formulation of detergents.



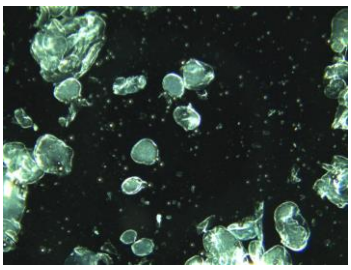
Fourteen lipolytic fungal strains were isolated from the sunflower oil industry by-products and were tested for its performance under laundry detergent components, considering the components of the commercial detergent TIDE™ as positive standard. Representative results and images from the fungal lipases evaluation process are presented.

Seven from the fourteen isolated fungal strain showed lipolitic activity when tested on minimum culture media agar plates amended with Rodamine and TIDE™ (previously enzyme inactivated) at the concentration provided by the manufacturer. A) *Penicillium* JC001 B) *Penicilium cpmmune*

JC44, C) *Penicillium* JC 045 D) *Penicillium* JC105 E) Penicillium JCCM" F) and G) *Aspergillus* R666 at 37 and 45 ºC respectively

Thermostability	
	From the 14 fungal strains JC R666 strain of the genus *Aspergillus*, which showed higher growth and lipolytic activity at 45 ° C.
Alkaline pH values	
	All strains were able to grow and show lipase activity at pH values from 8 to 9; however the strain JC 44 identified as *Penicillium commune* showed enzyme activity from pH8 to 11.
Surfactants stability	
	Once again the fungal strain *Penicillium commune* the highest growth and lipase activity when tested under: A) Sodium sulfate (9%)
	B) Sodium silicate (1%)
	C) Alkyl benzene sulfonate (0.5 %)
Lipolytic stability under laundry detergent conditions	

At the moment, studies in the culture media composition and fermentation process parameters are running in order to point out the best conditions for the fungal lipase to be used in laundry detergent formulation.



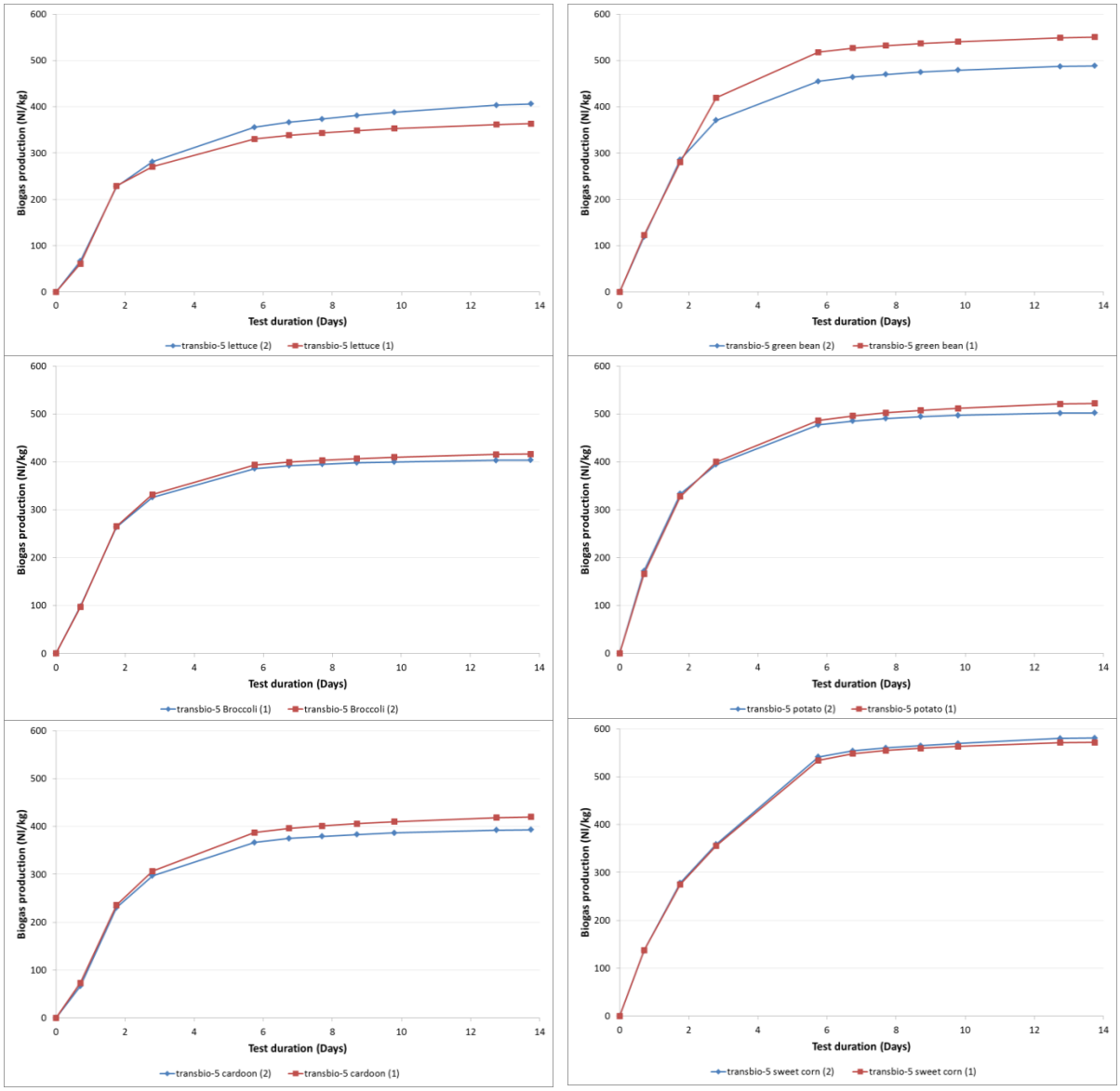
Proteos Biotech will check in **WP11** the enzymes purified from the solid state fermentation of different product and will study the compatibility of the enzymes with the most useful ingredients used in detergent and test **different ways to stabilize the** enzyme in the case that the selected enzymes aren't compatible with any component.

As a first step, Proteos will check the potency of the enzymes in order to value the optimal percent of the enzyme into the formulation. In a second step, a stability test of the enzyme and the detergent will be made in order to test that enzymes maintain their properties without modified any characteristic of the detergent. In a third

step, tests at lab scale will be performed with different temperatures in order to determine the best work temperature in a washing cycle.

These tests will help to choose the best enzymes to be applied in a detergent and different formulations of the detergents will be selected. With these enzymes, Tecnalia will be able to start the semi-scale test and check the enzymes in a real washing machine cycle.

In **WP12** six by-products (broccoli, cardoon, green bean, lettuce, potato and sweet corn), which form the start material for the different investigated processes in Transbio, were tested for their biogas potential. All materials showed a fast degradability and good biogas productions. Although the test materials were previously milled and dried, the results indicate that no long retention times are needed. The highest biogas production was obtained for sweet corn. The final goal is to check if organic by-products of the Transbio processes can be treated by anaerobic digestion, yielding electricity and fertilizer. The filter cake of sweet corn by-product after hydrolysis showed a little lower degradability compared to the original sweet corn by-product, but still 78% of all organic matter is converted to biogas, while methane content is similar to the untreated by-product. These first results indicate that the sweet corn filter cake after hydrolysis is an interesting substrate for anaerobic digestion. In next period more by-products of the Transbio processes will be evaluated and continuous lab-scale trials will be conducted. A model will be calculated to evaluate full-scale anaerobic digestion.



The graphics above shows the evolution of the biogas potential of broccoli, cardoon, green bean, lettuce, potato and sweet corn by-products

WP14 aims at conducting an environmental and economic impact study of the novel biotechnological approaches. For this purpose first an initial situation analysis was made in order to construct a reference scenario for comparison with the new TRANSBIO products. From this analysis it was concluded that fruit and vegetable wastes are currently mainly used for energy or fertilizer purposes and not necessarily for bioproducts. A literature analysis also revealed that traditional PHB, enzymes and succinic acid production induce a fossil fuel saving, but it is a general impression that information on these pathways is still very limited and that the used mass and energy balances depend on a lot of assumptions and projections. In this work package also the first preliminary environmental assessment results are obtained of PHB produced in the TRANSBIO project through cutting, milling, drying, hydrolysis, PHB production and downstream processing. It was concluded that PHB yield and drying (+other energy) requirement are the most determining factors for the environmental sustainability of this production pathway.

News

Cantanhede, 24 April 2014

The President of the European Commission, Mr. José Manuel Durão Barroso, visited the facilities of TRANSBIO partner Biotrend, after presiding over the ceremony of the inauguration of a new building of Biocant park, the first technological park and incubator fully devoted to biotechnology.

MEET the Partners

Partner Biotrend will attend the following events where you can meet our colleagues and find out more about current project developments:

- July 1-2, 2014: Summer Course "Biorefinery and Industrial Applications, Santander, Spain
- Sep 24-26, 2014: Biospain, Santiago de Compostela, Spain
- Oct 1-2, 2014: European Forum on Industrial Biotechnology 2014, Reims, France

1st TRANSBIO Workshop – Meet us in Costa Rica

Objectives:

- raise awareness in the agro industrial sector of the Latin American Region on the value of biomass produced by agro industrial processing and its biotechnological applications

- stimulate technology transfer in the Latin American Region to academic and professionals of biotechnological sector regarding production of PHB, succinic acid and enzymes for detergent applications.

- offer a field trip to a company who is producing banana and pineapple to showcase by-products that could be used for biotechnological applications as biomass.

Expected participation: 35 people (maximum of 50 attendees).

Profile of participants: Researchers and students of UCR that are working in biotechnology and the use of agroindustrial biomass and Professionals from the Food Industry (producers of biomass or involved in biotechnological activities).

Dates: October 14 and 15, 2014

Location: CITA University of Costa Rica, San José, Costa Rica

PROGRAM

Tuesday October 14 (Basic concepts of biotechnology)

Schedule	Presentation	Speaker
7:00 – 13:00	Field Trip (Mundimar - Guácimo, Limón)	
13:30 – 13:45	Registration for Workshop	
13:45 – 14:00	Welcome	Thomas Dietrich TRANSBIO coordinator
14:00 – 14:30	Introduction to biotechnology	Lilia Arely Prado – UAM Mexico
14:30 – 15:30	Microbiology and fermentations used in biotechnology applications	Raquel Virto – CNTA, Spain
15:30 – 15:50	*Coffee Break*	
15:50 – 16:20	Use of agroindustrial biomass and its preparation for biotechnology	Xavier Roca – Promic S.A, Spain
16:20 – 17:00	Recovery procedures from fermentation broths and scaling up process	María Pilar Castro – Proteos, Spain
17:00 – 17:10	Conclusions	Thomas Dietrich

Wednesday October 15 (TRANSBIO project and biotechnological value added products)

Schedule	Presentation	Speaker
8:00 – 8:30	Registration	
8:30 – 8:40	Welcome	Thomas Dietrich TRANSBIO coordinator/ Carmela Velazquez - CITA, Costa Rica
8:40 – 9:00	General TRANSBIO project presentation	Thomas Dietrich TRANSBIO coordinator/ Tecnalia, Spain
9:00 – 9:45	Market opportunities and new value streams from renewable raw materials and wastes	Bruno Sommer Ferreira – Biotrend, Portugal
9:45 – 10:10	Cooperation / funding opportunities – HORIZON 2020, Regulatory incentives for biobased products	Bianca Pop, TRITECC, Romania
10:10 – 10:30	Coffee Break	
10:30 – 11:00	Biomass availability in Latin America /Costa Rica	Carmela Velázquez – CITA, Costa Rica
11:00 – 11:30	Challenges and opportunities - pre-treatment and hydrolysis or no pre-treatment	Christian Marie Bols – Wetlands Incubator, Belgium
11:30 – 12:00	Autochthon strain selection for value added applications by biotechnology	Celia Sacramento Santos Pais – Universidade do Minho, Portugal
12:00 – 12:30	Fermentation process in agitated submerged tanks bioreactors – (PHB and Succinic acid production)	Raquel Virto, CNTA, Spain
12:30 – 13:30	Lunch	
13:30 – 14:00	Fermentation process in solid state bioreactors	Holmer Wöhlk, NST, Germany
14:00 – 14:30	Enzyme Application in detergent formulations	María Pilar de Castro, Proteos, Spain
14:30 – 15:00	How to recover added-value products after fermentation of your residues	Jessica Wildner, ttz Bremerhaven, Germany
15:00 –15:20	*Coffee break*	
15:20 – 15:50	Biogas production from biomass and fermentation by-products: from waste to energy	Steven Verstichel, OWS, Belgium
15:50 – 17:10	Roundtable: Why waste is still waste? What are we doing with our waste? How can waste become "money"?	Industry, Academy and Government participants *Moderator:* Carmela Velázquez, CITA, Costa Rica
17:10 – 17:20	Conclusions	Thomas Dietrich, Tecnalia, Spain, TRANSBIO Coordinator

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