Pilot scale biotransformation of vegetal oil into natural green notes flavor using sugar beet leaves as sources of hydroperoxide lyase

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Introduction

Natural green note aromas (GLVs) are highly attractive flavors commonly used in the food industry. These are produced in extremely low levels upon physiological stress in plant organs of any sort. This weak sporadic presence entails a very expensive extraction step to obtain pure GLVs. Therefore catalytic biotransformations of fatty acid sources, the initial substrate for GLVs, have been developed. Enzymatic defense pathways and particularly the LOX pathway produce the major part of GLVs. Unlike GLV molecules that are emitted in the atmosphere, the enzymes are extractible from the plant material. Thus, a combination of plant enzyme extracts and substrate preparations provides all the ingredients for GLV production. Besides, sugar beet leaves present high levels of hydroperoxide lyase among plant sources and are available in large amounts during three months. In this enzymatic pathway, fatty acids are successively transformed by lipase, lipoxygenase and hydroperoxide lyase into aldehydes and alcohols, final compounds of GLVs pathway. Limiting and problematic steps occur with the action of hydroperoxide lyase, when enzymatic catalysis is followed by an enzyme destabilization. Alternative substrates bind irreversibly to the heme group of the enzyme and end the reaction. This poster briefly describes the development of a complete bioprocess for natural GLV production, from hydrolysis to purification. A high level of biotransformation could be achieved using optimum experimental conditions and a cheap source of plant materials.

Objective and purpose

Establishing a simple production bioprocess of green leaf flavors using unwanted plant materials like sugar beet leaves. The process has to be efficient and leads to the purest flavor composition.

Material and Methods

All reactions have been performed in pilot scale vessels, including mixed bioreactor of 100L. Reaction order simply follows the natural lipoxygenase pathway present in most of the superior plants.

Main substrate source

Sugar beet leaves do not present high levels of fatty acids content (only 0,1%). Therefore, the use of an external source of initial substrate is necessary. Vegetal oils are cheap sources of unsaturated fatty acids which are easily obtained, and stable enough for flavor production. Final aroma composition will depend on concentration of linoleic or linolenic acid present in the oil.

	Free Fatty Acid Content		Potential C6-GLVs production	
Oil Type	C18:2	C18:3	Hexanal	Hexenal
Sunflower oil	61%	3%	196	80
Safflower oil	78%	2%	251	5
Linseed oil	16%	54%	50	170
Kiwifruit oil	5%	62%	17	197

Tab 1. Synthesis potential for different vegetal oils related to their maximum level of C_6 Aldehydes production (mg/g of oil)

These oils are highly valorized through this bioprocess because this aroma is sold at more than one hundred times the price of the oil. But only C_{18} unsaturated acids of the oil are metabolized through this enzymatic pathway.

Plant materials characterization

Nowadays sugar beet leaves are a unvalued by-product of the agricultural industry in Europe. This raw plant material presents valuable enzymatic content, like hydroperoxide lyase activity. Specific characterization of the potential GLVs production for these enzymes has led to the following result.

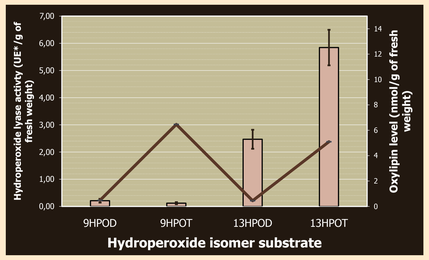


Fig. 1 : Degrading hydroperoxide activity in sugar beat leaves. Bars represent the hydroperoxide lyase activity related to the four hydroperoxide substrates. Lines show the concentration of corresponding hydroperoxide isomer in the leaves.

The hydroperoxide substrates naturally present in leaves are clearly linolenic acid derivate : 9-HPOT and 13-HPOT. Although concentrations are very low, this indicates that sugar beet have preferentially developed this way of synthesis. Furthermore, only 13-hydroperoxide lyase activity has been found. Thus, Z-3-Hexenal and E-2-Hexenal, resulting from the activity of 13-hydroperoxide lyase on 13-HPOT, are the preferential compounds we are going to investigate for flavor production with sugar beet leaves.

Fatty acids hydrolysis

Following plant characterization, linseed oil has been chosen as substrate source, on account of its high linolenic acid content. Hydrolysis is a common chemical reaction in industry. We hydrolyze the oil enzymatically with lipase to keep neutral condition at room temperature. Hydrolysis is performed by immobilized lipases from a Bacillus strain. Enzymes are recycled 3 times and a high yield (more than 90%) of free fatty acids has been recovered after centrifugation. No emulsifying compounds have been used to avoid loss of free fatty acids by centrifugation.

Fatty acids oxidation

The lack of LOX activities (less than 0,1 UE*/ g of fresh weight) in sugar beet leaves requires an external source of this enzyme. Actually, crude soybean seeds are the best sources of 13-lipoxygenase activity. This reaction is fast and completed within 30 minutes at 4°C and pH 9.3.

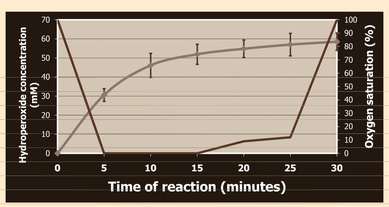


Fig. 1 : Biotransformation of 100mM linolenic acids solution into hydroperoxide by 13-Lipoxygenase. Grey line represents the hydroperoxide concentration during reaction, and brown line the oxygen level in bioreactor.

Aldehyde synthesis

This is the most critical step of the lipoxygenase pathway which is due to the instability of hydroperoxide lyase activity. The aldehyde synthesis has to be carefully controlled. After the setup of the optimum conditions (pH and temperature) for the hydroperoxide lyase of sugar beet, ground plant materials are quickly added to the production juice containing previously synthesized hydroperoxides.

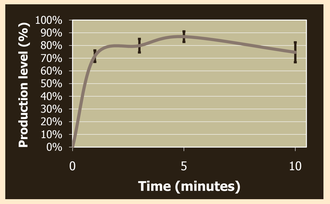


Fig. 3 : Evolution of the global aldehyde level within the first ten minutes after the addition of sugar beet leaves

Following the mixing of substrates and enzymes, only productions of Z-3-Hexenal and E-2-Hexenal were monitored. Maximum reaction levels are obtained within 5 minutes and thereafter decrease slowly due to the aldehyde consumption by other enzymatic activities from the plant materials. The maximum concentration can be maintained by pH reduction to inhibit the enzymatic degradation.

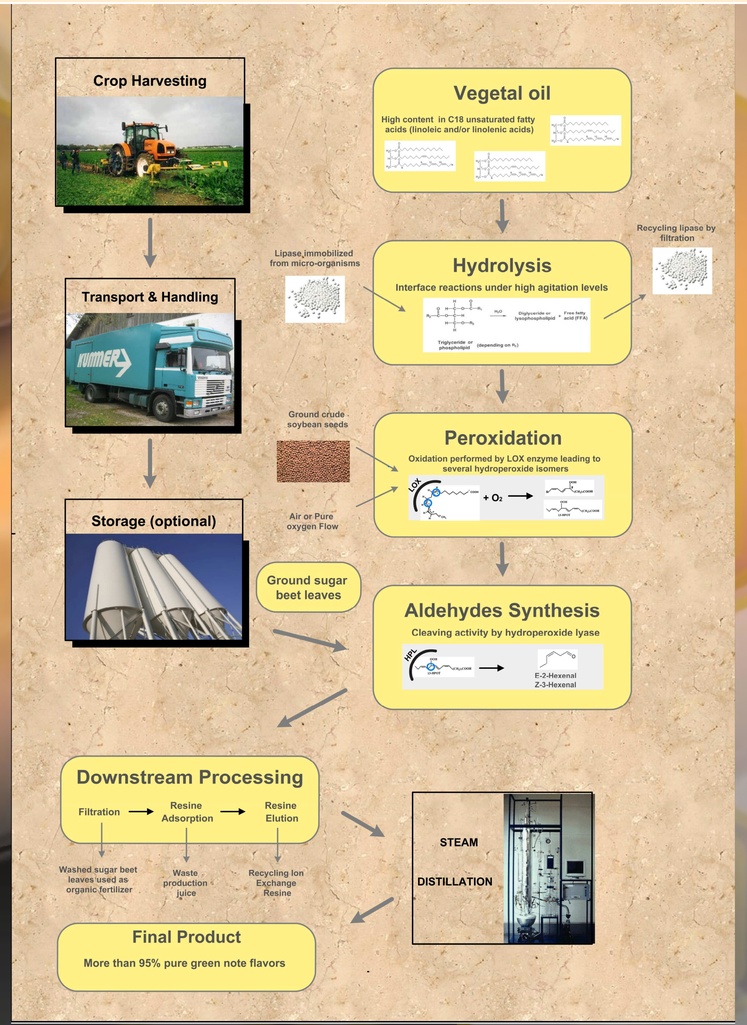
Fig. 4 : Evolution of productivity (grey) and yield (brown) levels of Z-3-Hexenal related to 13-hydroperoxide substrate concentration.

High GLVs concentrations are difficult to obtain because of the inhibition of hydroperoxide lyase above 0,15 mM of hydroperoxide substrate. But further experiments with substrate concentrations above 2mM have shown better productivity levels in spite of a lower yield.

Downstream Processing

After the synthesis steps, an optional alcoholic conversion of aldehydes into alcohols can be performed, but in our case no transformation was made. Firstly, the production juice is cleared of plant fragments, by filtration through 0.5mm filters upon 1 bar of pressure. To recover aldehydes from the juice, adsorption on ionic resin has been adapted. The MN-202 resin from purolite, usually used for water cleansing, presents perfect characteristics and cheap prices to accomplish the adsorption. 95% of the aldehydes are effectively adsorbed and after that 75% are eluted in isopropanol or a different alcoholic solvent. Secondly, steam distillation is used to purify the flavor juice by evaporating alcohol. No azeotropic gas was observed during distillation and all aldehydes could be conserved. But, our apparatus was not able to separate hexanal from hexenal isomers. Also, during distillation it was observed that all Z-3-Hexenal isomers were totally converted into E-2-Hexanal.

Proposed pilot-scale biotransformation process of vegetal oil into green note flavor using sugar beet leaves



Conclusion

At the end of the process, pure aldehydes (Hexanal and E-2-Hexenal) can be recovered. These compounds can be labeled as "natural flavor" because they are vegetal oil derivates. The price value is more than 1000$/kg for these compounds and they could be easily sold in food and beverage sectors. This long and complex biotransformation of cheap resources into expensive flavors may be a solution to convert unwanted crop byproducts, such as sugar beet leaves.

* 1UE corresponding to 1mM of hydroperoxide converted by minutes