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Conversion of winery wastes into a sugar-rich hydrolysate for the biotechnological production of succinic acid

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European Union
European Regional Development Fund

HELLENIC REPUBLIC
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EPAnEK 2014–2020
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COMPETITIVENESS ENTREPRENEURSHIP INNOVATION

ΕΣΠΑ 2014-2020
ανάπτυξη - εργασία - αλληλεγγύη
Partnership Agreement 2014 - 2020

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Objectives

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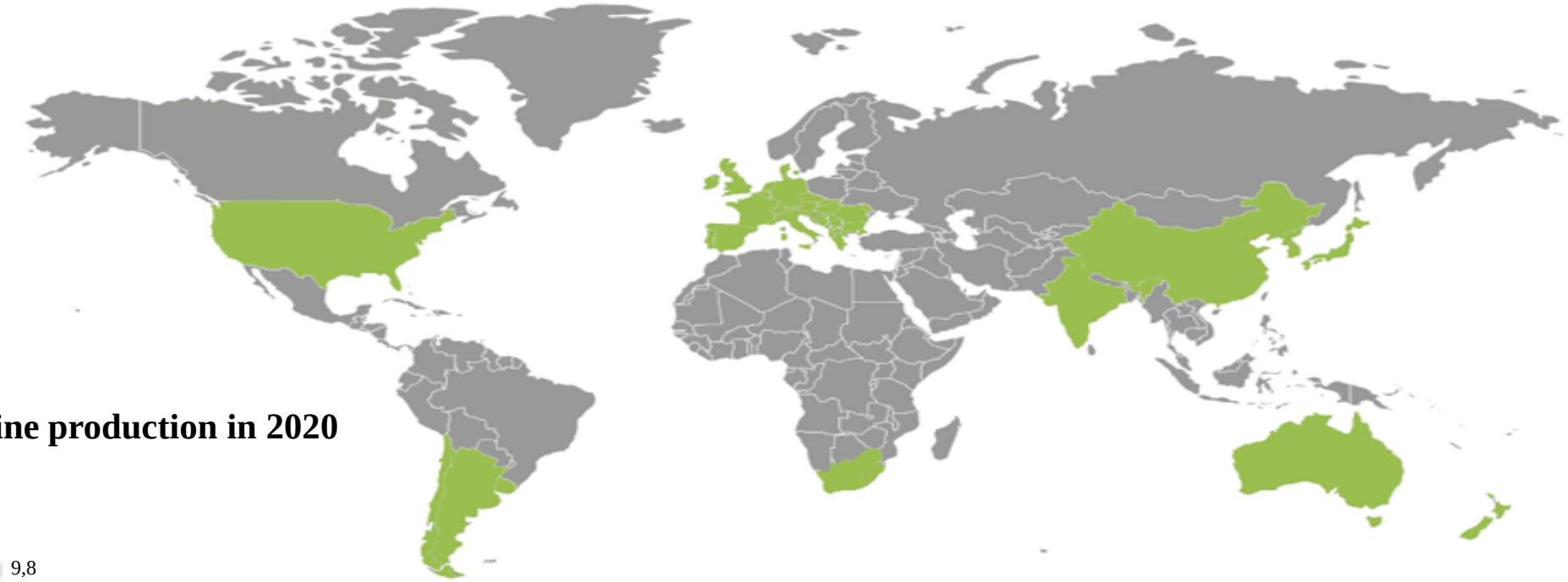
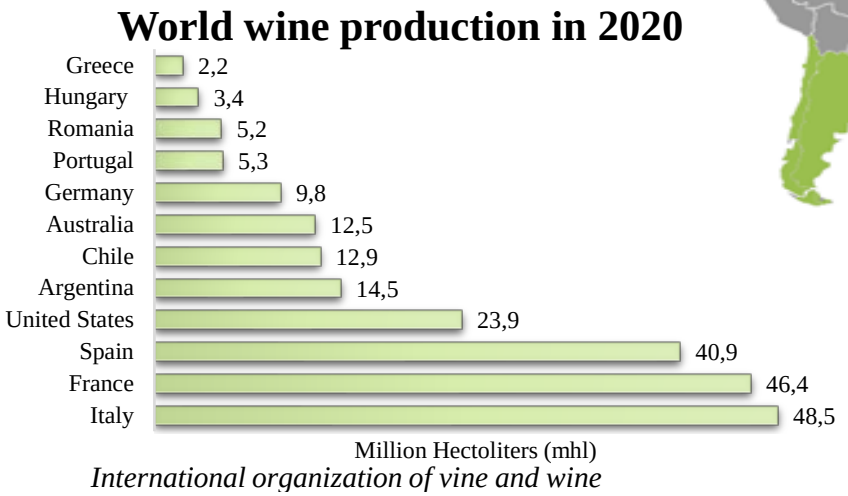
- Renewable raw materials – winery by-products
  - Grape pomace
  - Grape stalks
- Development of an integrated biorefinery for the valorisation of grape pomace and stalks generated after the winemaking process of the Greek variety Agiorgitiko for:
  - The production of bio-based succinic acid
  - The extraction of value-added fractions (lipids and phenolic compounds)
- Optimisation of enzymatic hydrolysis of pretreated grape pomace and stalks
- Evaluation of different feeding strategies in the fermentation process

Global wine production

Wine making worldwide production of 260 million hectoliters in 2020





International organization of vine and wine

By-products produced from the wine making process

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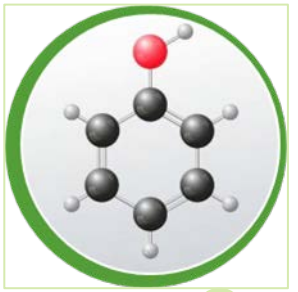
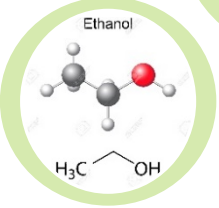
Wine making process

Vinification by-products

Process development

Products & Applications





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Bio-based Succinic Acid

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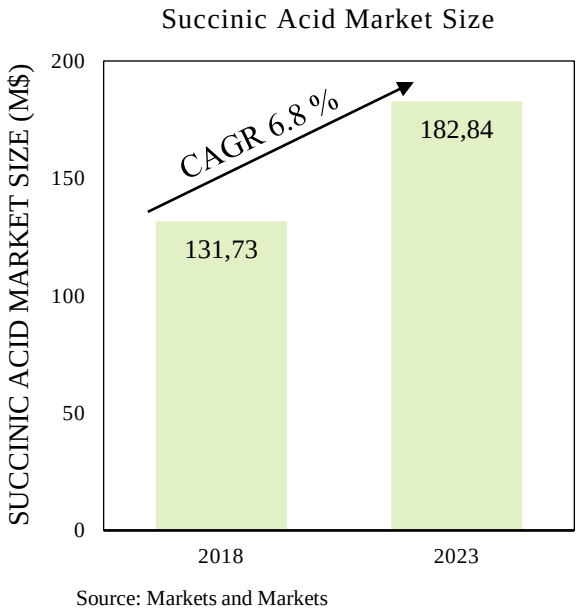
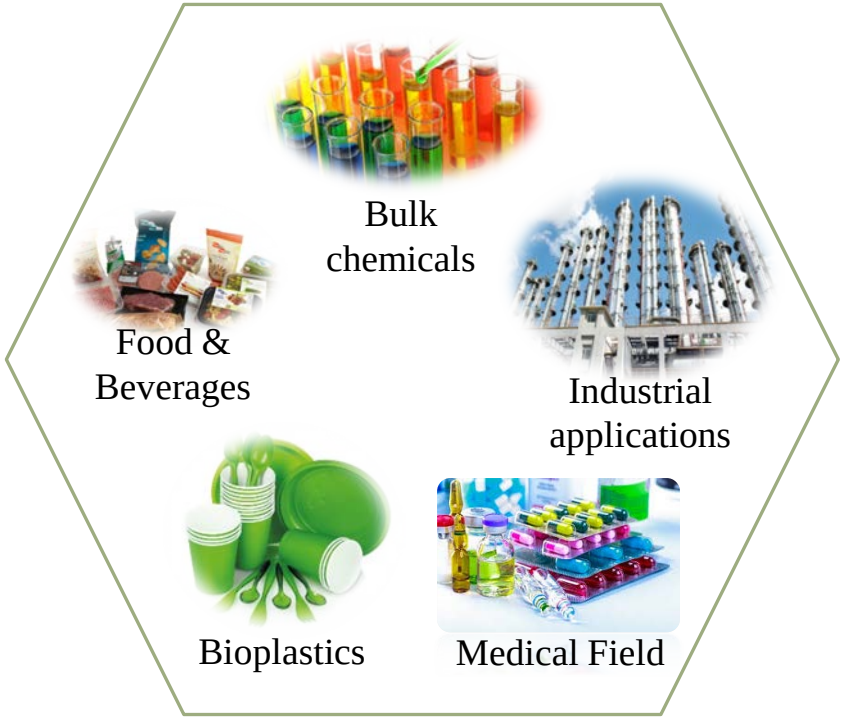
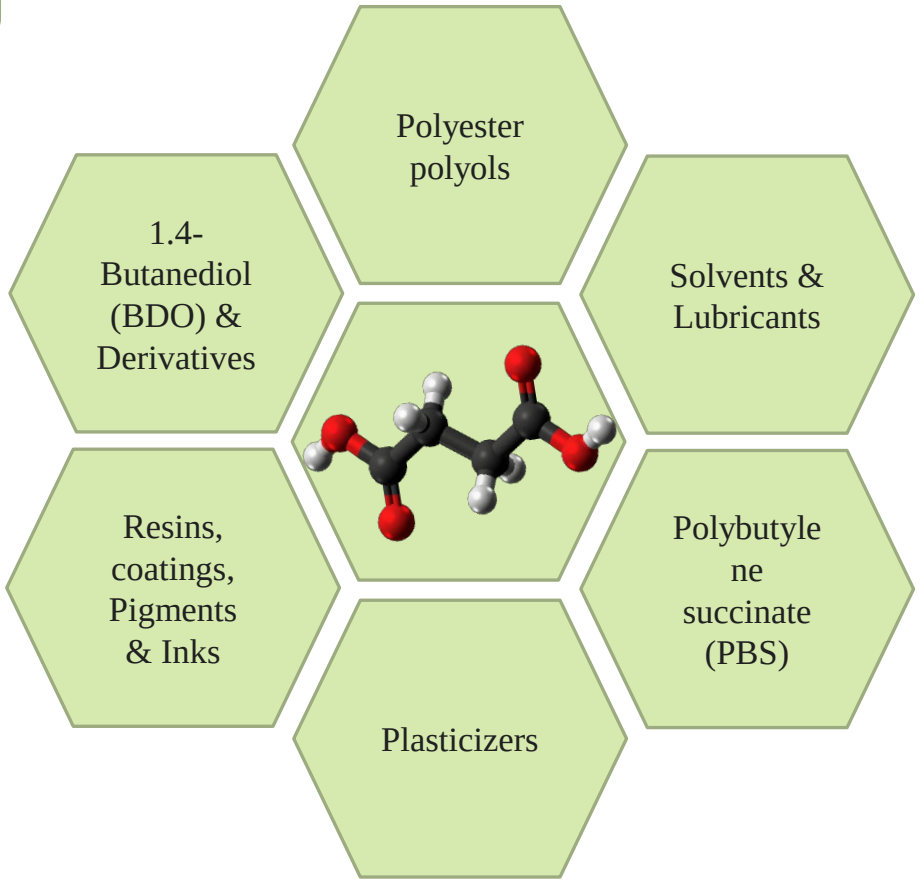
Succinic acid is one of the top 12 high-value-added bio-based chemicals
Intermediate in several chemical processes



Source: Markets and Markets



Industrial Applications



*CAGR: Compound annual growth rate

Composition of Agiorgitiko Pomace & Stalks

Components (%, dry basis)	Pomace	Stalks
Free sugars	2.55 ± 0.03	5.44 ± 1.17
Protein	9.36 ± 0.50	7.12 ± 0.26
Lipids	8.20 ± 0.07	8.07 ± 0.73
Ash	4.79 ± 0.13	6.76 ± 0.10
Lignin	34.79 ± 0.70	29.73 ± 0.59
Cellulose	20.90 ± 0.75	21.65 ± 0.01
Hemicellulose	13.31 ± 0.53	15.25 ± 0.88
Tannins	2.62 ± 0.02	0.74 ± 0.09
TPC (g GAE)	2.47 ± 0.05	1.36 ± 0.07
Ethanolic extract presents:		
TPC (mg GAE/ g extract)	185.75 ± 3.75	57.34 ± 4.71
Antioxidant Activity Index	1.03 ± 0.12	0.47 ± 0.02

Free sugars, cellulose and hemicellulose may provide carbon sources for succinic acid production

TPC: Total Phenolic Content
GAE: Gallic Acid Equivalents

Process flow diagram

Grape pomace

Water extraction
1/10 w/v, 2h, 45 ºC

Free sugars

Residual solids

Hexane extraction
1/10 w/v, 2h, 25 ºC

Lipids

Residual solids

Grape stalks

(1:1 ratio)

Extraction with
EtOH 70% – 0.5%
HCl 0.1 M

Phenolic - rich crude extract

Alkaline treatment
NaOH 1%, 1/10 w/v, 3h, at 100 ºC

Residual solids

Acid hydrolysis
H_2SO_4 3%, 1/4 w/v, 121 ºC
for 40 min

Residual solids

Enzymatic hydrolysis
(cellulase, β-glucosidase, BSA)

sugar - rich hydrolysate

Fermentation with A. succinogenes

Succinic Acid

Composition of Agiorgitiko pomace and stalks mixture before alkaline treatment	
Lignin (%, dry basis)	34.32
Cellulose (%, dry basis)	18.72
Hemicellulose (%, dry basis)	15.20
Protein (%, dry basis)	11.05
Extractives (%, dry basis)	15.40

Enzymatic hydrolysis conditions

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Pomaces & stalks from Agiorgitiko → *Delignification & Acidic hydrolysis* → Residual solids → *Enzymatic Hydrolysis* → Glucose rich fraction

Alkaline pretreatment (1% (w/v) NaOH, 100 °C for 3 h):

- ✓ Lignin removal 52% (w/w)

Dilute acid pretreatment (3% (v/v) H_2SO_4, 121 °C for 40 min):

- ✓ Hemicellulose solubilisation 53% (w/w)

Composition of the remaining solids (on dry basis):

- ✓ Cellulose: 42.38 %
- ✓ Hemicellulose: 10.89 %
- ✓ Lignin: 29.79 %

Enzymes and enzyme activities (Units / g substrate)
20 FPU Celluclast* & 80 U β-glucosidase
20 FPU Celluclast & 40 U β-glucosidase
10 FPU Celluclast & 10 FPU Viscozyme & 80 U β-glucosidase
10 FPU Celluclast & 10 FPU Viscozyme & 40 U β-glucosidase

* Cellulase from *Trichoderma reesei*

The addition of 8 mg BSA/g dry sample was also tested using all of these enzyme combinations

Conditions:

- ❖ 50 mM sodium citrate buffer (pH 5)
- ❖ Temperature: 50 °C
- ❖ Solid to liquid ratio: 10%
- ❖ Under mechanical stirring for 72 h

BSA: Bovine Serum Albumin

Optimisation of Enzymatic Hydrolysis

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Composition (%, dry basis): 42.38 Cellulose, 10.89 Hemicellulose, 29.79 Lignin

20 FPU/g substrate Celluclast

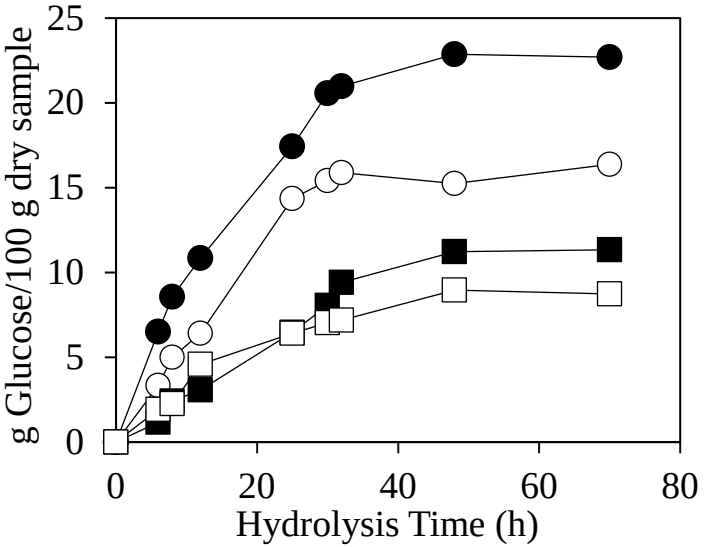
and

● 80 U β-glucosidase /g substrate & BSA

○ 80 U β-glucosidase /g substrate

■ 40 U β-glucosidase /g substrate & BSA

□ 40 U β-glucosidase /g substrate



Cellulose-to-glucose conversion yield:

● 0.48 g/g

○ 0.35 g/g

■ 0.24 g/g

□ 0.19 g/g

10 FPU/g sub. Celluclast & 10 FPU/ g sub. Viscozyme

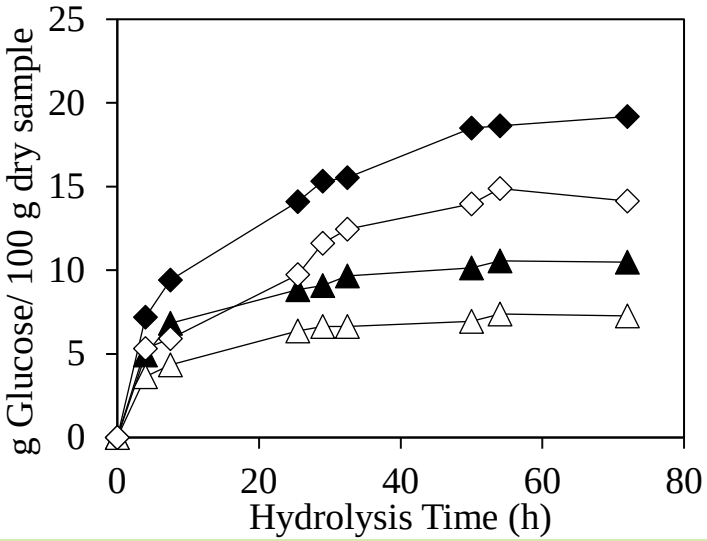
and

♦ 80 U β-glucosidase /g substrate & BSA

◊ 80 U β-glucosidase /g substrate

▲ 40 U β-glucosidase /g substrate & BSA

△ 40 U β-glucosidase /g substrate



Cellulose-to-glucose conversion yield:

♦ 0.41 g/g

◊ 0.30 g/g

▲ 0.22 g/g

△ 0.15 g/g

Optimisation of Enzymatic Hydrolysis

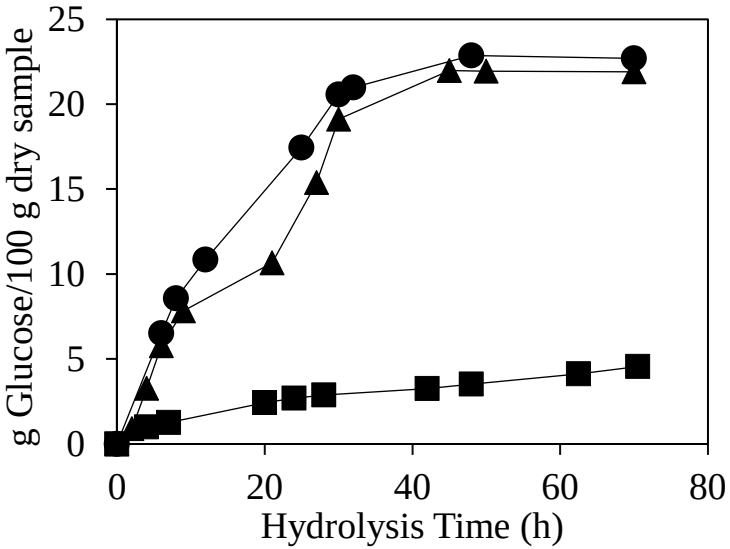
The best enzyme combination considering glucose release from cellulose was evaluated using:

- ❖ either deionized water or
- ❖ the direct addition of the enzymes to the acid hydrolysate

20 FPU/g substrate Celluclast, 80 U β-glucosidase /g substrate & BSA

Enzymatic hydrolysate solution:

- ● Citrate buffer
- ▲ Deionized water
- ■ Acid hydrolysate



Cellulose-to-glucose conversion yield:

- ● 0.48 g/g
- ▲ 0.44 g/g
- ■ 0.10 g/g

Succinic Acid Production

Pomaces & stalks from Agiorgitiko → *Delignification & Acidic hydrolysis* → Residual solids → *Enzymatic Hydrolysis (Cellulase 20 FPU/g substrate β-glucosidase 80 U/g substrate, BSA)* → Glucose rich fraction

- ✓ Strain: *Actinobacillus succinogenes*
- ✓ Carbon Source: Enzymatic hydrolysate (22.7 g/L glucose)
- ✓ Feeding: Concentrated free sugars from Agiorgitiko pomaces (consisting mainly of glucose and fructose)
- ✓ Nitrogen source: 5 g/L Yeast Extract
- ✓ $MgCO_3$: 5 g/L
- ✓ Working volume: 500 mL
- ✓ Temperature: 37 ºC
- ✓ pH: 6.7
- ✓ Aeration: CO_2 0.1vvm
- ✓ Agitation: 180 rpm

Batch & Fed-Batch Fermentations

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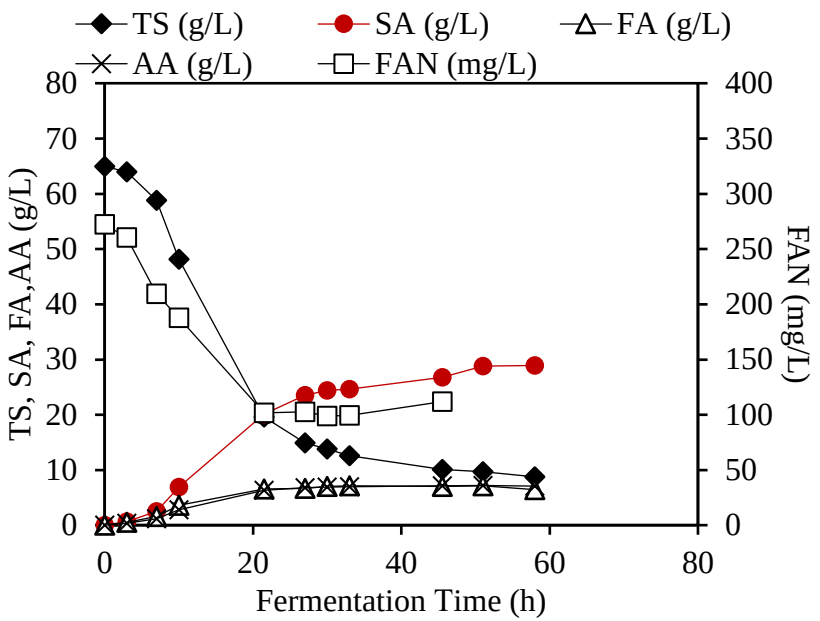
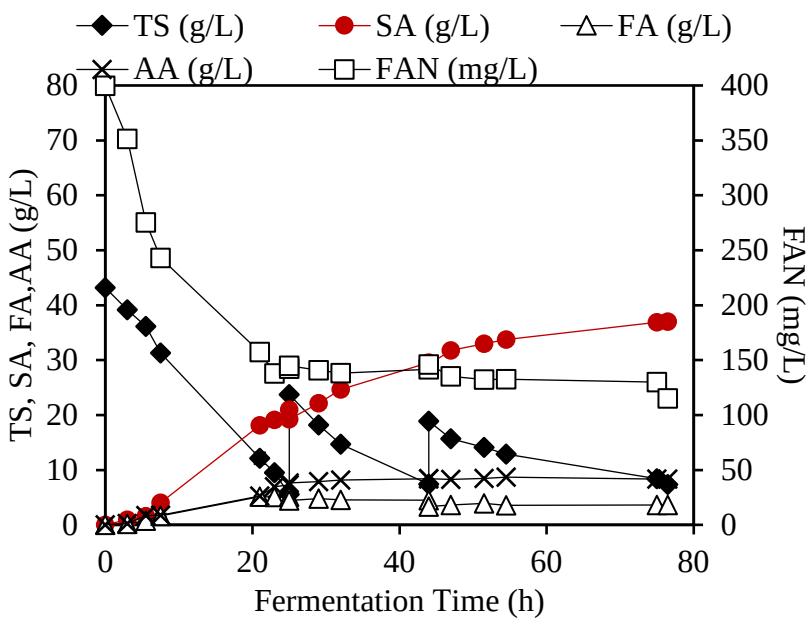
Batch Fermentation



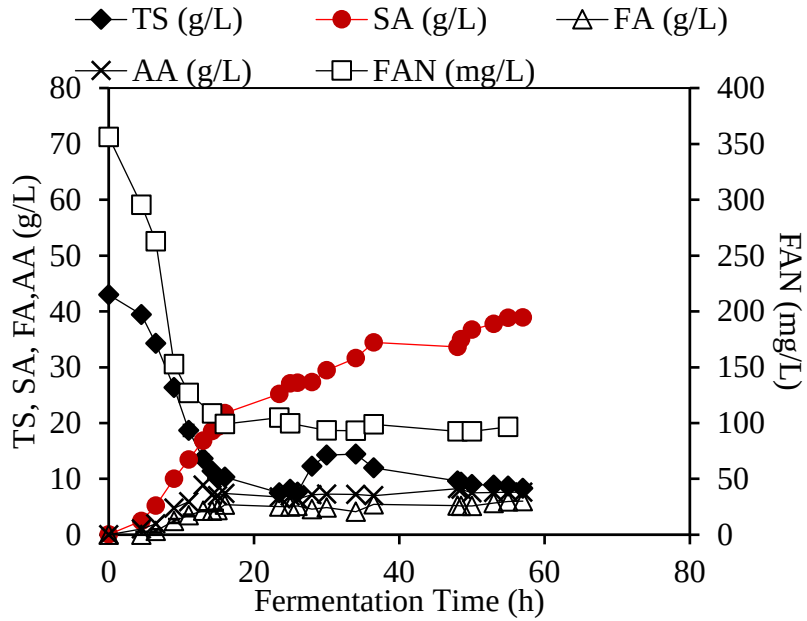
Succinic Acid (SA): 24.6 g/L
Formic Acid (FA): 7.1 g/L
Acetic Acid (AA): 6.9 g/L
Yield*: 0.47 g/g
Productivity: 0.75 g/(L·h)

Fed batch fermentation, Feeding with pulses



Succinic Acid (SA): 37.0 g/L
Formic Acid (FA): 3.6 g/L
Acetic Acid (AA): 8.3 g/L
Yield*: 0.68 g/g
Productivity: 0.48 g/(L·h)

Fed-batch fermentation, Continuous feeding

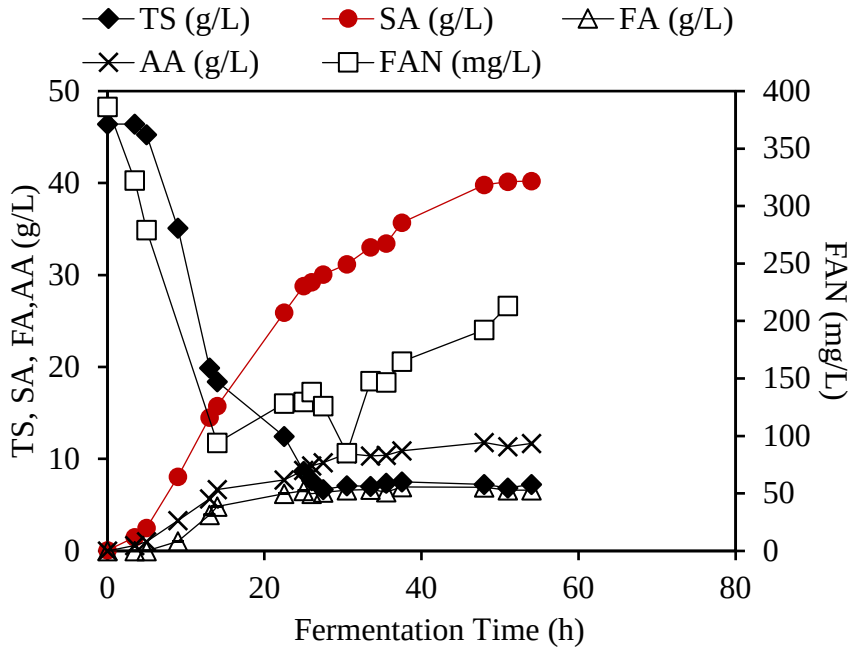


Succinic Acid (SA): 37.8 g/L
Formic Acid (FA): 5.7 g/L
Acetic Acid (AA): 7.6 g/L
Yield*: 0.67 g/g
Productivity: 0.71 g/(L·h)

*yield was calculated as the grams of sugars consumed divided by the total grams of succinic acid produced during fermentation

Fed-batch fermentation, with YE in feeding solution

Continuous Feeding supplemented with yeast extract (10%)



Succinic Acid (SA): 40.2 g/L
Formic Acid (FA): 6.6 g/L
Acetic Acid (AA): 11.7 g/L
Yield*: 0.67 g/g
Productivity: 0.79 g/(L·h)

Fermentation type	Fermentation time (h)	SA (g/L)	Yield (g/g)	Productivity (g/(L·h))	FA:SA	AA:SA	Total by-products: SA
Batch	33	24.6	0.47	0.75	0.29	0.28	0.57
Fed-batch with pulses	76.5	37.0	0.68	0.48	0.10	0.23	0.32
Fed-batch with continuous feeding	53	37.8	0.67	0.71	0.15	0.20	0.35
Fed-batch with continuous feeding & YE	51	40.2	0.67	0.79	0.16	0.29	0.46

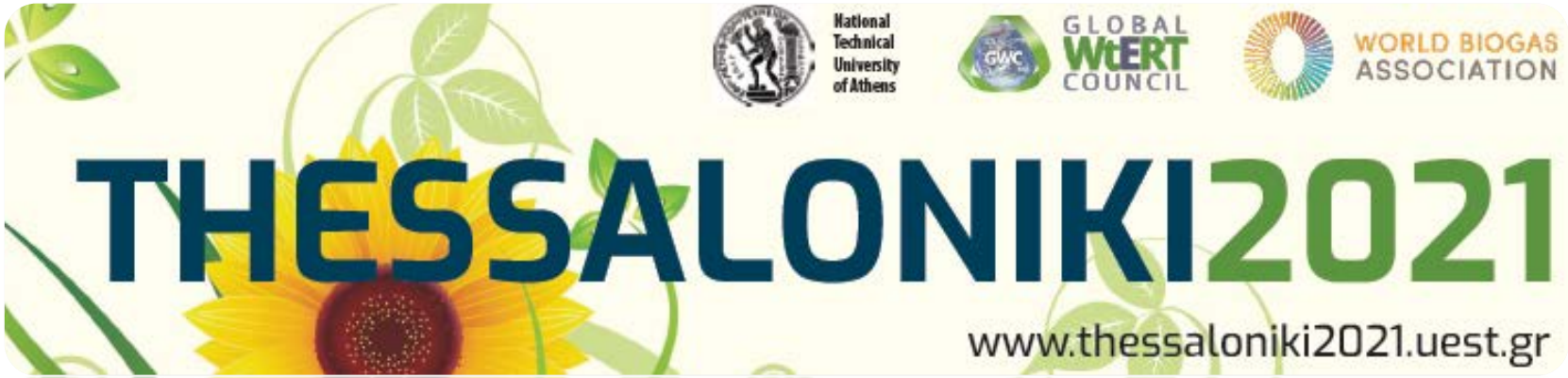
*yield was calculated as the grams of sugars consumed divided by the total grams of succinic acid produced during fermentation

Conclusions

A novel process was developed utilising waste streams produced after the winemaking process

- Value-added components (lipids, antioxidants) were isolated from the by-product streams
- The hydrolysis of the pretreated material was successfully performed without the addition of buffer solution
- The addition of BSA in enzymatic hydrolysis acts positively
- Fed-batch fermentations with continuous feeding improved succinic acid production
- The addition of yeast extract in feeding solution increases the concentration of succinic acid, productivity and by-product formation

The valorisation of winery wastes from Agiorgitiko variety leads to the development of sustainable wineries



THANK YOU FOR YOUR ATTENTION!

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