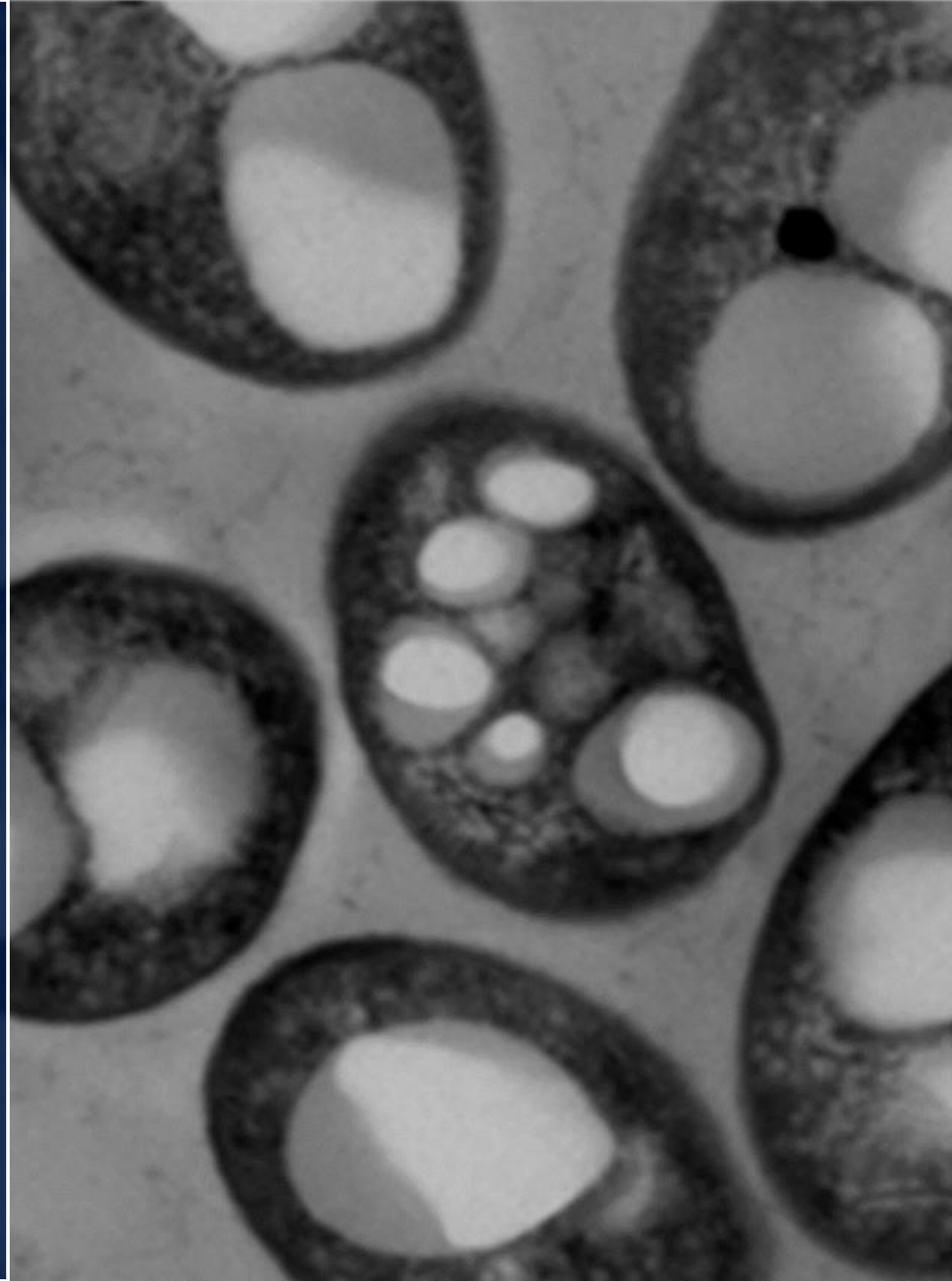
Production of Eco-Friendly Polyhydroxyalkanoates Using Waste Starch

Authors: Karlo Grgurević, Dajana Kučić Grgić, Martina Miloloža, Vesna Ocelić Bulatović



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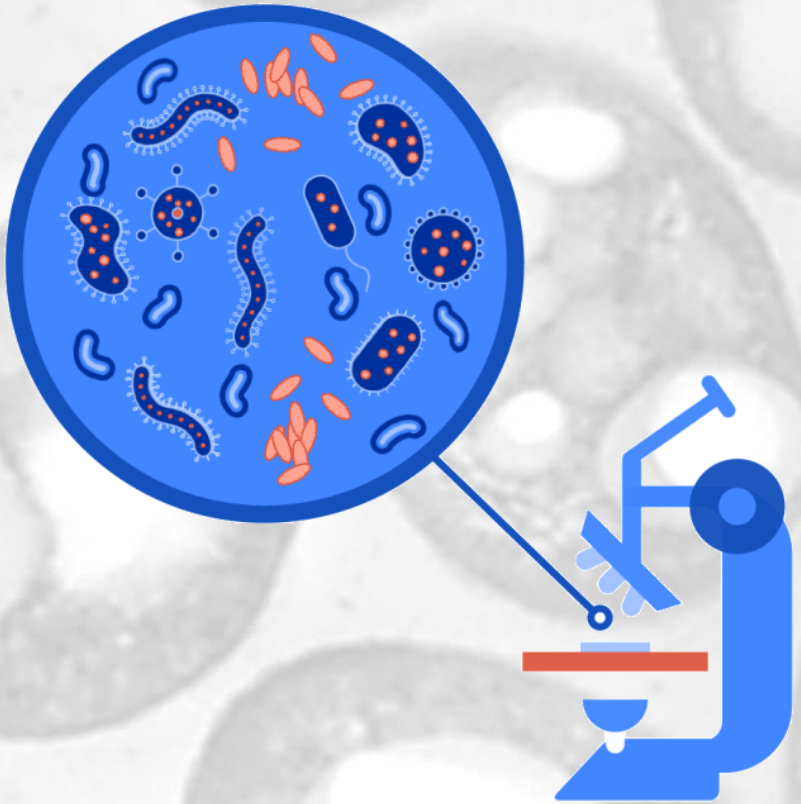


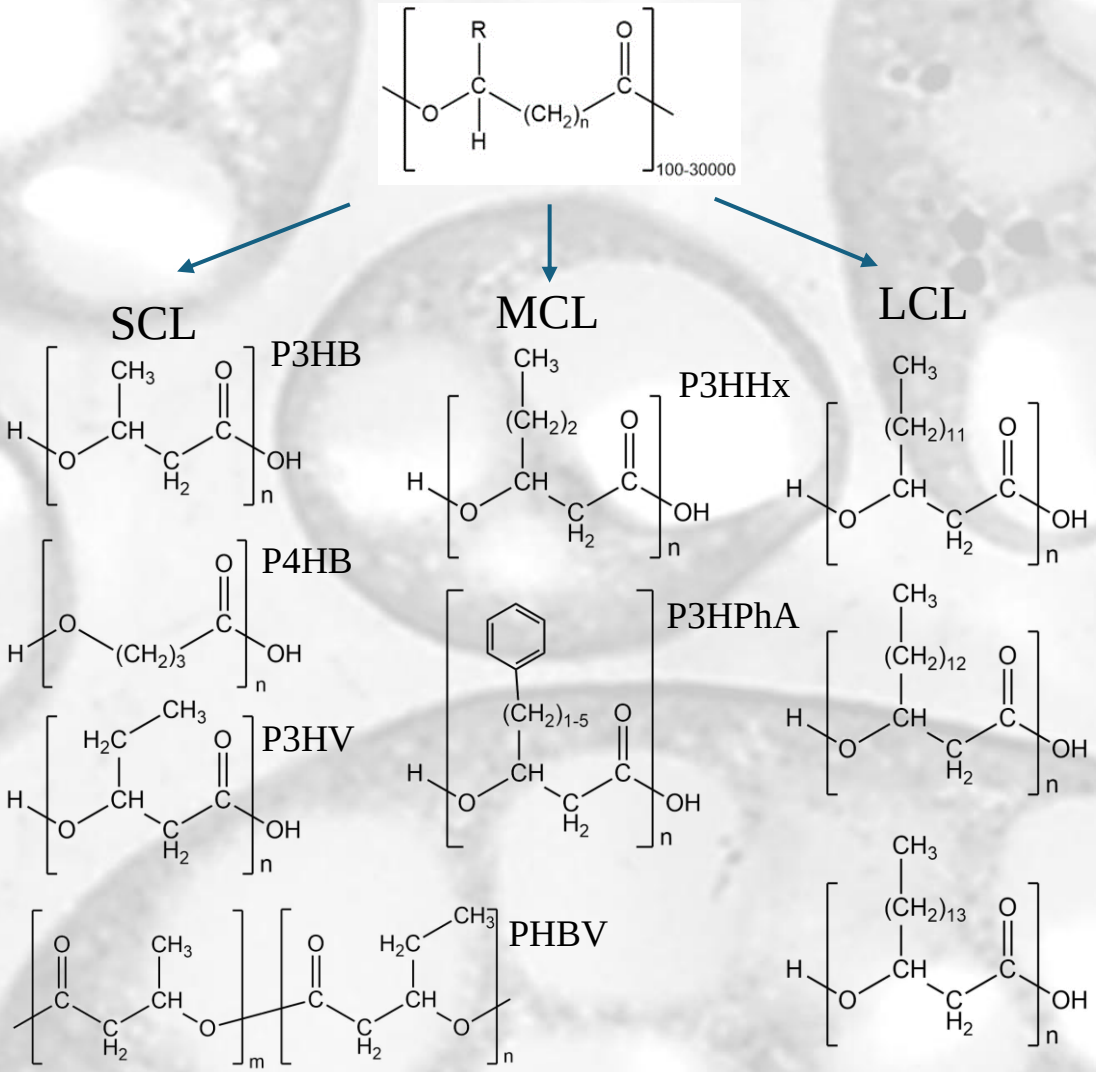
1. Introduction

- synthetic polymers – problems
- biodegradability
- polyhydroxyalkanoates (PHA)
- agro-industrial waste– pretreatment
- fermentation

Oil rafinery

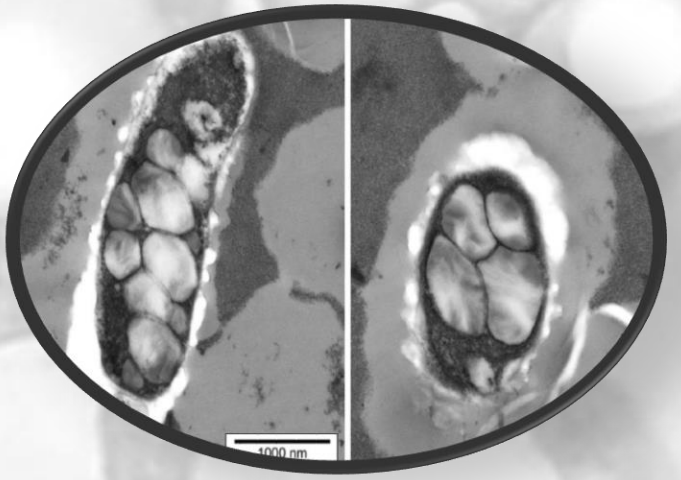
Plastic waste accumulation

1.1. Polihydroxyalkanoates, PHA



PHA classification

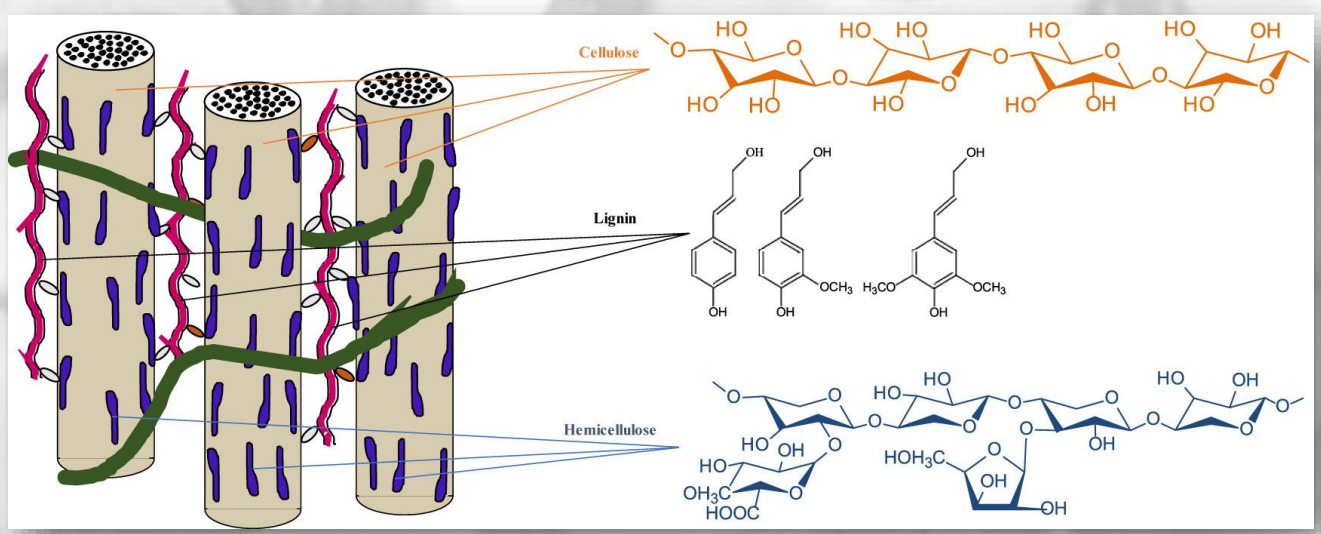
- biodegradable polyesters
- microorganisms in stress conditions
- Martinus Beijerinck (1888), Maurice Lemoigne (1923)
- 150 different PHA kinds
- P3HB & P4HB



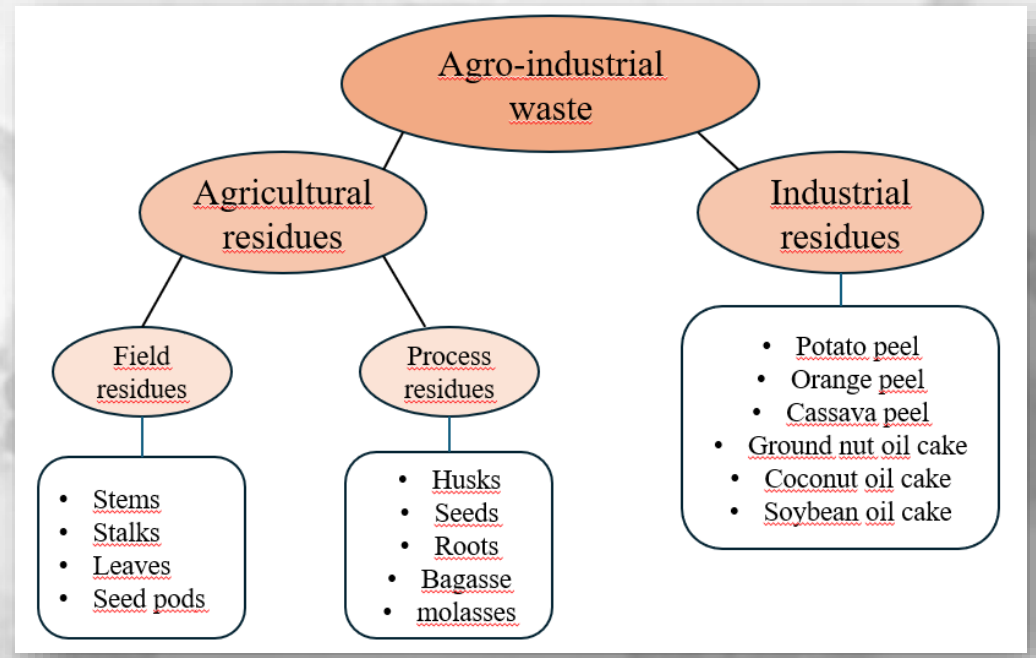
SEM photography of PHA granule in microorganisms' cells

1.2. PHA production from agro-industrial waste in microorganism cell

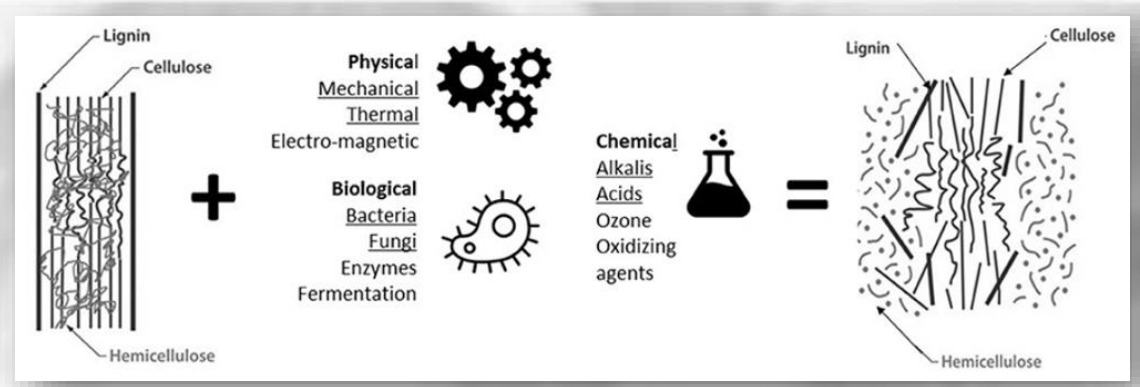
- lignocellulose – cellulose, hemicellulose, lignin
- various nutrients→ supstrate adequate for fermentation process
- pH, temperature, moisture, reducing sugars content, C:N
- pretreatment is necessary



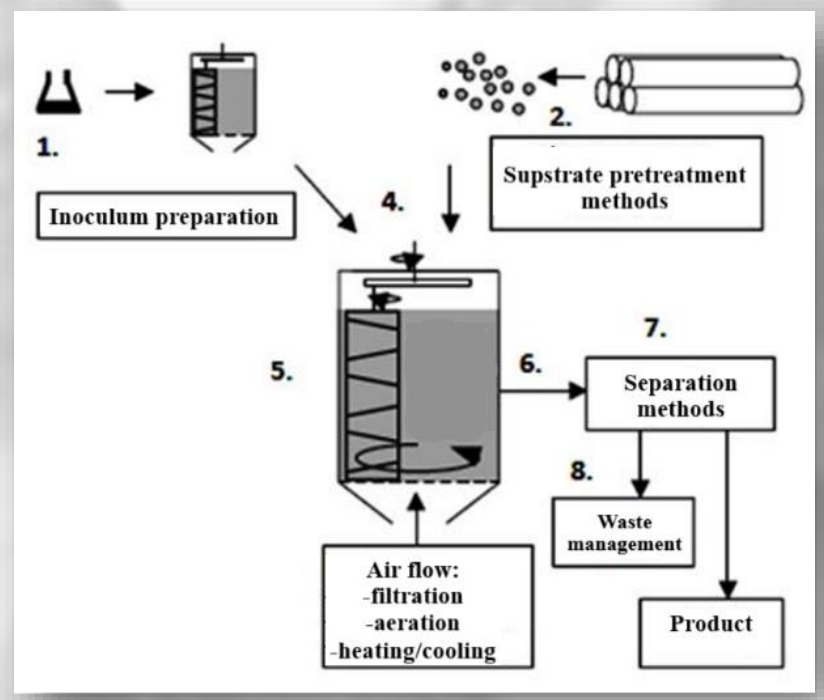
Lignocelulose structure



Agro-industrial waste classification

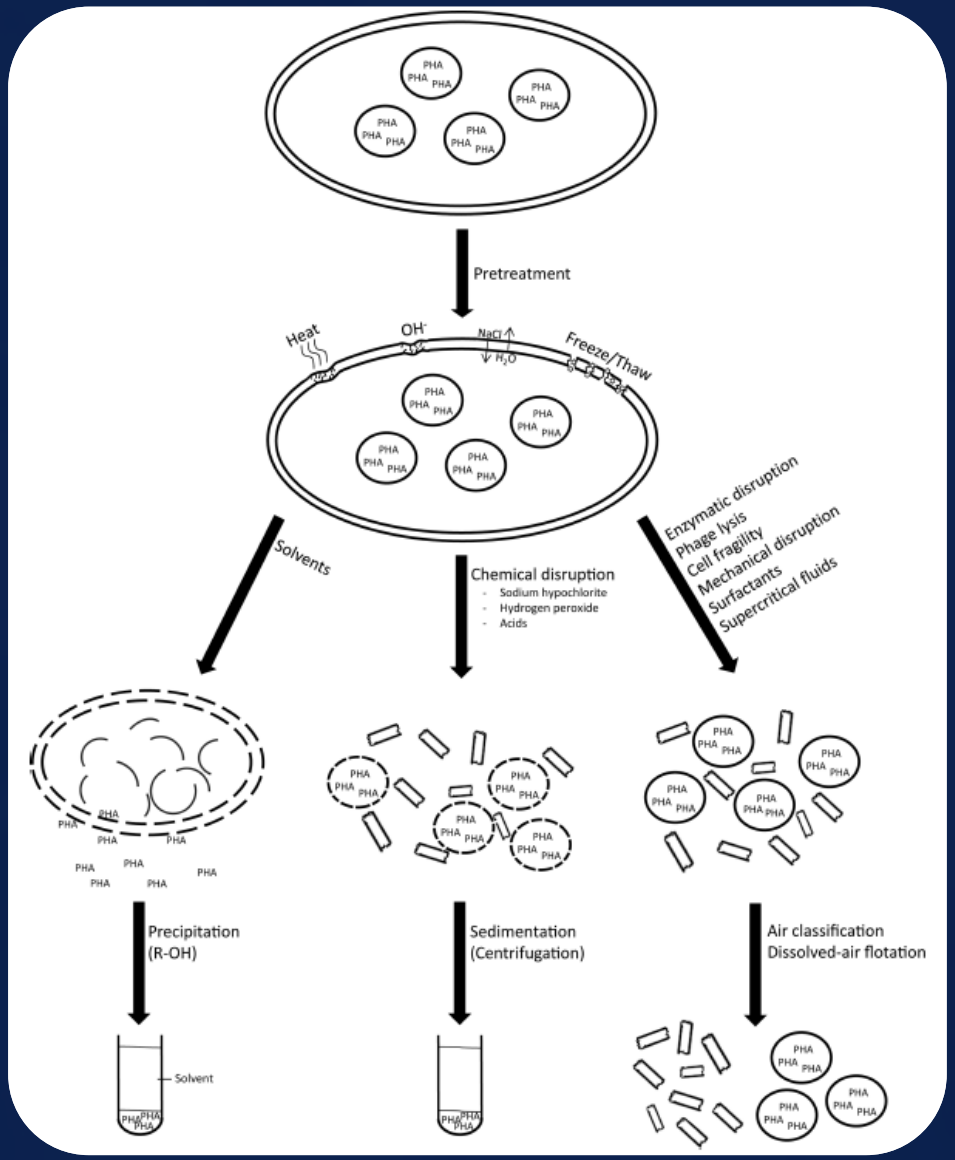


Waste pretreatment



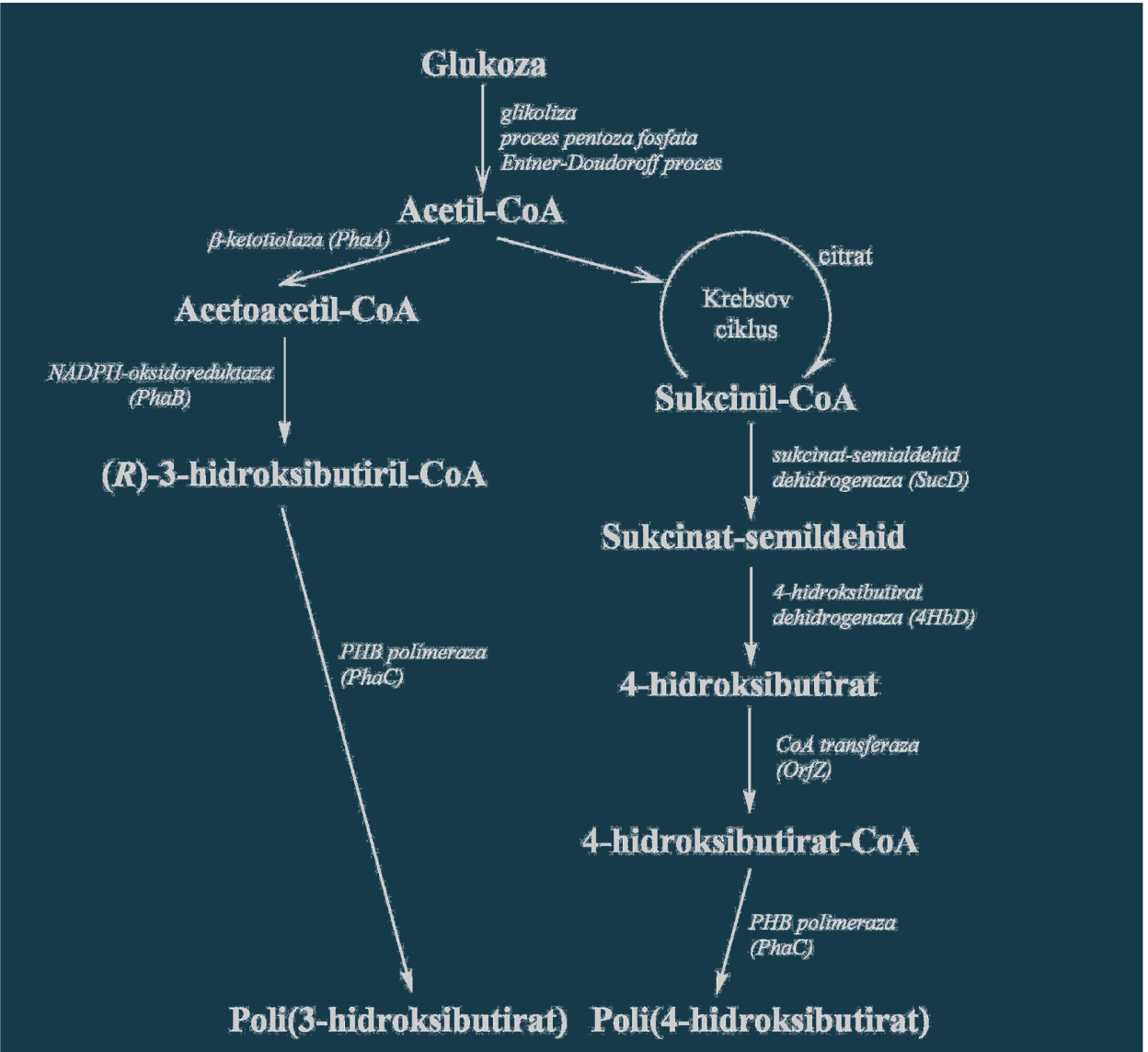
„Solid – state fermentation" process

- pretreatment – lignocelulose degradation, specific area increase, particle size decrease…
- SSF – batch reactor
- cell separation→ cell disruption→ PHA extraction

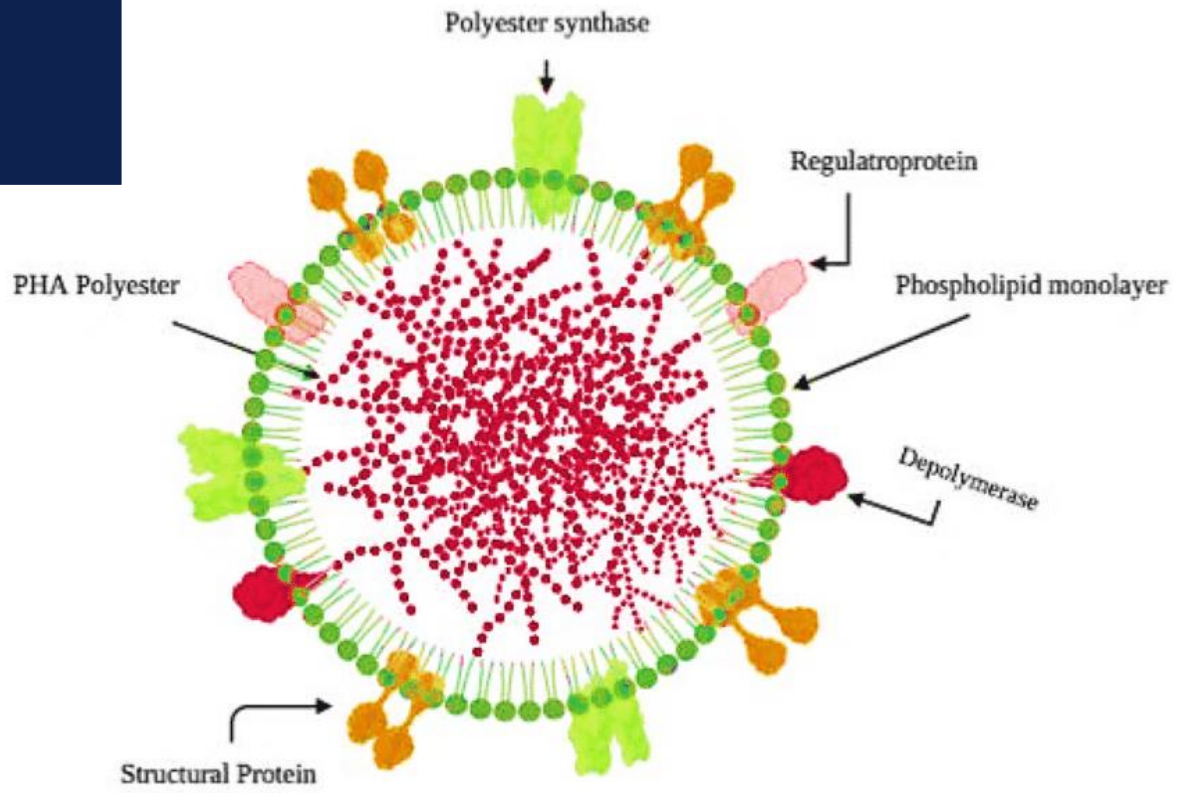


Cell separation and disruption, PHA extraction

2.3. PHA biosynthesis

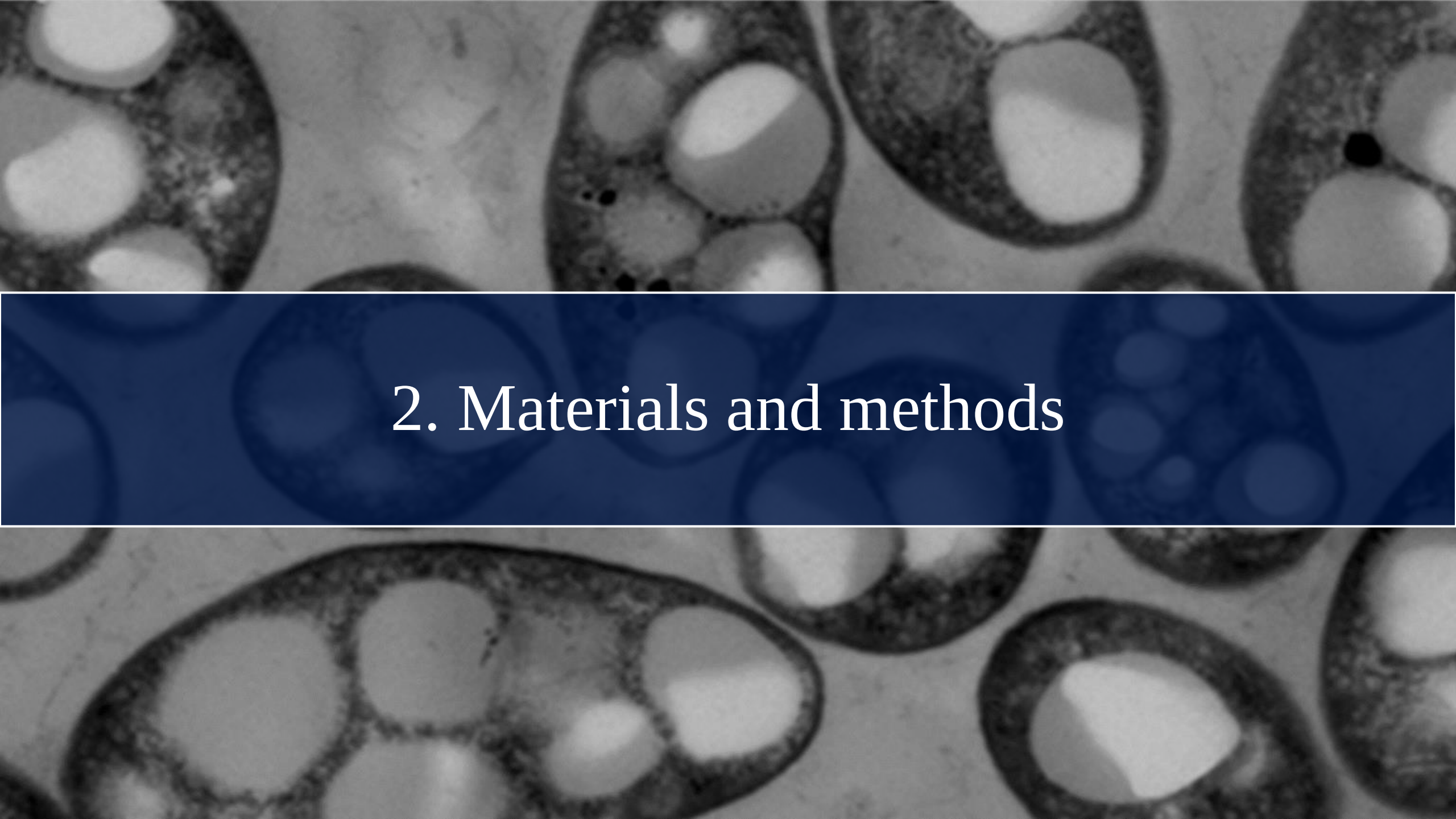


Biochemical pathways for PHA accumulation from glucose



Makrostruktura granule PHA

- excess of carbon, low levels of nitrogen, phosphorus…
- reserve energy source
- PHA granules (inclusions)
- genetically modified microorganisms
- bacteria, fungi and mixed cultures



2. Materials and methods

Waste starch characterisation

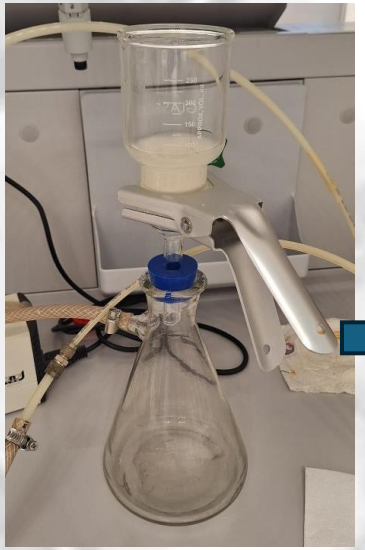
Moisture, dry matter and volatile matter content determination

Reducing sugar content determination

Microorganism isolation and identification

Waste starch

Starch pretreatment process optimisation and PHA production



Ultrasound (US) pretreatment od starch (US power, pretreatment time and NaOH concentration)

„Solid-state" fermentation

PHA extraction

FTIR

TGA

DSC

Exp.	Time of US / min	US power / W mL^{-1}	*c* (NaOH) / M
1	30	1	0,01
2	30	1	0,05
3	30	1	0,1
4	30	2	0,01
5	30	2	0,05
6	30	2	0,1
7	30	3	0,01
8	30	3	0,05
9	30	3	0,1
10	60	1	0,01
11	60	1	0,05
12	60	1	0,1
13	60	2	0,01
14	60	2	0,05
15	60	2	0,1
16	60	3	0,01
17	60	3	0,05
18	60	3	0,1
19	90	1	0,01
20	90	1	0,05
21	90	1	0,1
22	90	2	0,01
23	90	2	0,05
24	90	2	0,1
25	90	3	0,01
26	90	3	0,05
27	90	3	0,1

Waste starch characterisation

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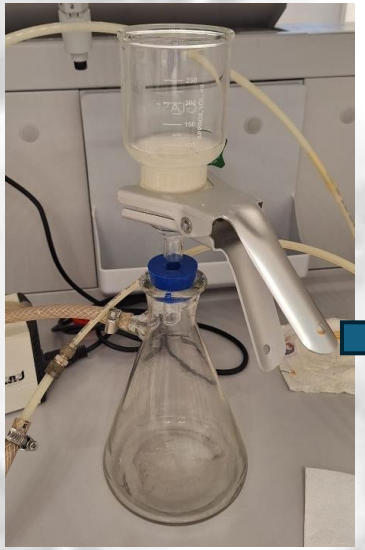


Ultrasound (US) pretreatment od starch (US power, pretreatment time and NaOH concentration)

„Solid-state" fermentation

PHA extraction

FTIR

TGA

DSC

4. Results and discussion

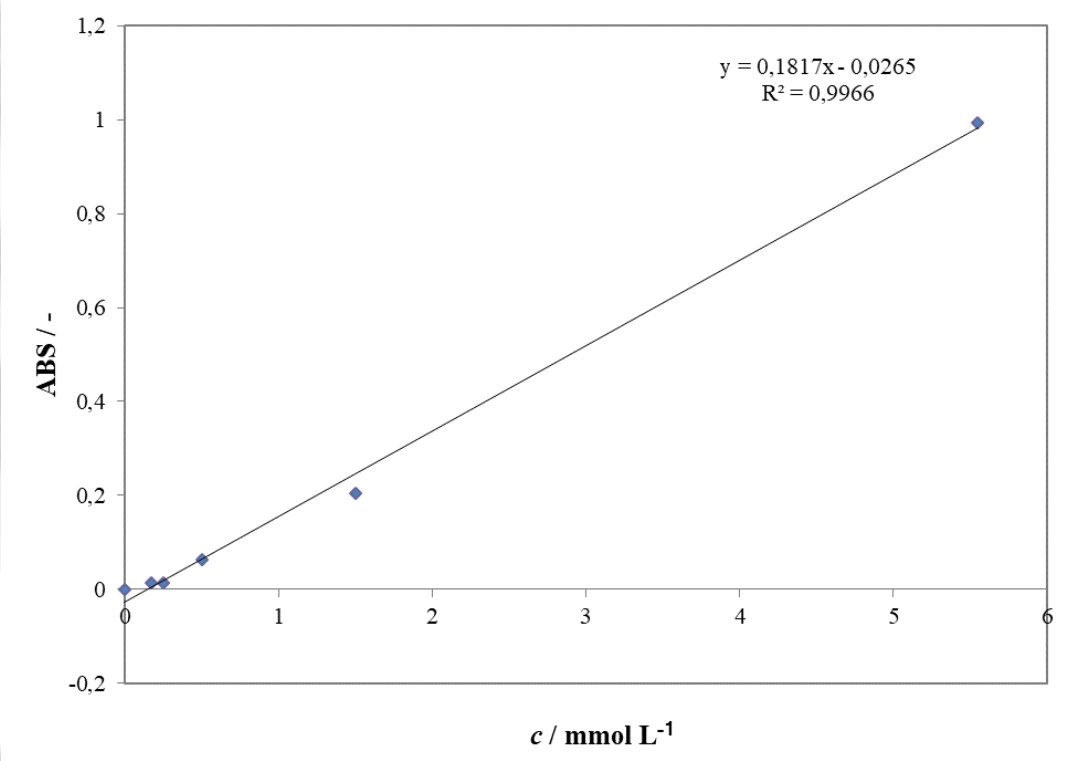
3.1. Waste starch characterisation

Table 3.1. Initial values of moisture and dry and volatile matter mass fraction in waste starch; pH and conductivity.

Sample	w (H_2O) / %	w (DM) / %	w (VM) / %	pH-value	κ / µS cm^{-1}
Starch	44,77	55,23	99,36	4,453	433

Table 3.2. CFU values taken from different nutrient supstrates.

Microorganism	CFU / cells g_{DM}^{-1}
Bacteria (HA)	$1{,}45 \cdot 10^8$
Fungi (molds and yeasts) (SA)	$7{,}38 \cdot 10^6$
Bacteria and fungi (PHA)	$8{,}95 \cdot 10^7$



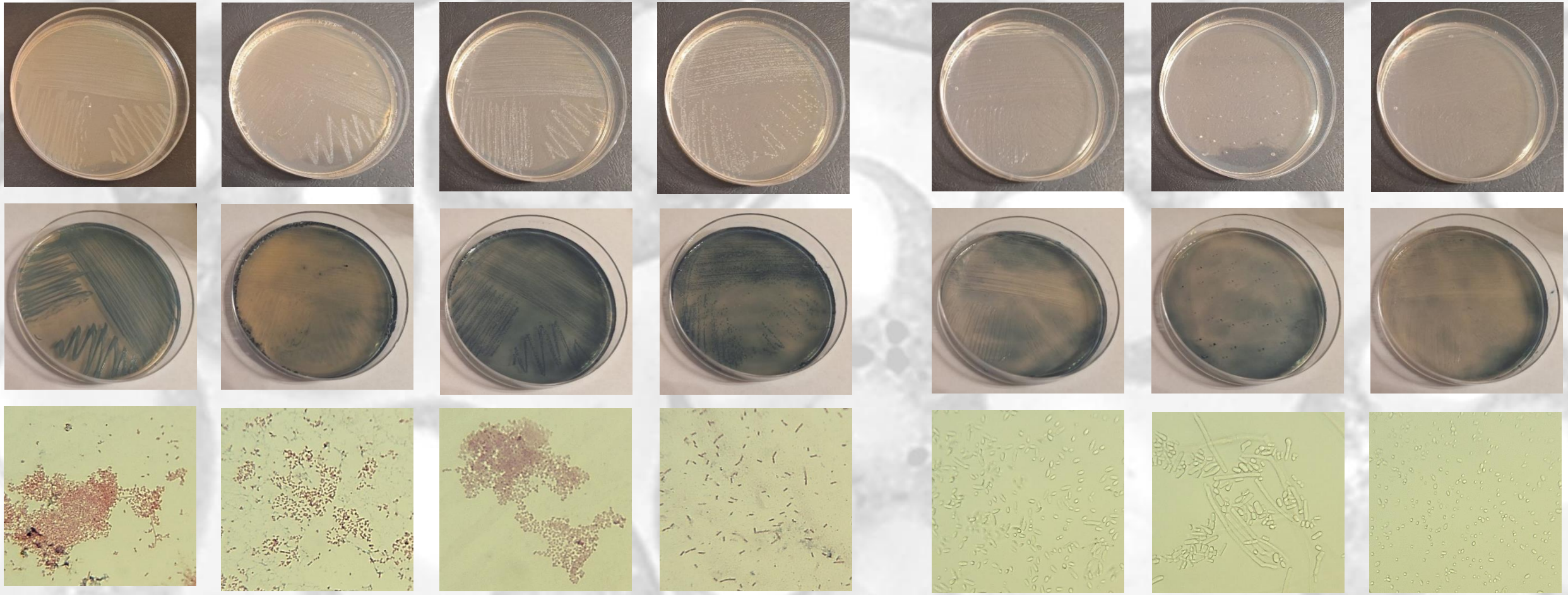
DNS calibration curve

c = 0,2253 mmol L^{-1}

3.2. Microorganism isolation and identification

4 bacteria

3 fungi



Leukonostoc sp. (79,9 %)

Citrobacter freundii (99,9 %)

Staphilococcus lentus (99,9 %)

Bacillus licheniformis (99,9%)

Cryptococcus humicola (88,9 %)

Geotrichum klebahnii (88,0 %)

Candida krusei (88,0 %)

3.3. Waste starch pretreatment optimisation

Pretreated starch characterisation

Table 3.3. Pretreated starch characterisation (before SSF process).

Exp.	$w(H_2O)$ / %	w(DM) / %	w(VM) / %	pH	κ / mS cm^{-1}	$\gamma(O_2)$ / mg L^{-1}	T / °C
1	14,60	85,40	99,65	9,590	0,206	8,07	22,6
2	14,42	85,58	98,74	10,256	1,693	8,11	23,3
3	19,30	80,70	97,79	10,385	2,480	8,08	23,5
4	14,53	85,47	99,68	9,807	0,379	8,02	23,8
5	18,00	82,00	98,85	10,214	1,541	7,97	23,9
6	35,99	64,01	98,23	10,373	2,200	7,96	23,8
7	19,12	80,88	99,50	9,845	0,404	7,90	23,8
8	43,13	56,87	99,05	10,236	1,557	7,95	24,1
9	58,47	41,53	98,78	10,034	1,726	7,58	24,2
10	16,72	83,28	99,56	9,733	0,282	8,08	23,1
11	18,08	81,92	98,66	10,262	1,742	8,10	23,1
12	26,28	73,72	97,92	10,270	1,553	7,99	23,6
13	17,72	82,28	99,57	9,534	0,315	7,83	24,0
14	17,65	82,35	98,71	10,107	1,438	7,86	24,2
15	64,56	35,44	97,66	10,152	1,995	7,48	24,5
16	16,68	83,32	99,68	9,508	0,215	7,63	24,7
17	18,27	81,73	98,78	10,147	1,605	7,87	24,2
18	72,45	27,55	98,95	10,017	1,520	7,56	24,4
19	17,48	82,52	99,61	9,425	0,357	7,97	23,8
20	17,69	82,31	98,66	10,038	1,665	7,92	24,0
21	10,24	89,76	97,52	10,343	3,010	7,94	24,0
22	16,41	83,59	99,63	9,228	0,319	7,91	24,1
23	17,29	82,71	98,78	9,908	1,600	7,89	24,0
24	76,12	23,88	98,59	10,035	3,090	7,85	24,1
25	15,97	84,03	99,66	10,261	0,288	7,79	24,3
26	16,53	83,47	98,75	10,033	1,691	8,02	24,0
27	13,01	86,99	96,66	9,898	1,780	7,82	23,9

Table 3.4. Initial CFU values of microbial suspension (cells mL^{-1}) and waste with added microbial suspension (cells g_{DM}^{-1}).

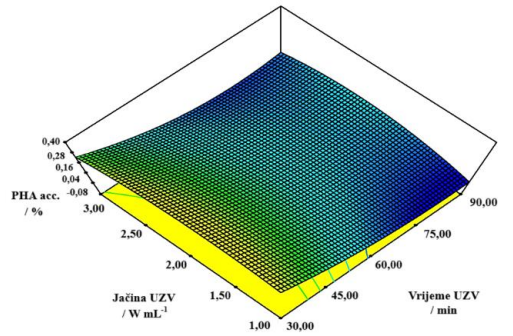
	Suspension (initial)		Suspension + waste (initial)	
	HA	**SA**	**HA**	**SA**
Exp.	CFU / cells mL^{-1}	CFU / cells mL^{-1}	CFU / cells g_{DM}^{-1}	CFU / cells g_{DM}^{-1}
1	$2{,}67 \cdot 10^9$	$1{,}97 \cdot 10^8$	$8{,}00 \cdot 10^7$	$1{,}16 \cdot 10^7$
2	$2{,}78 \cdot 10^9$	$3{,}57 \cdot 10^7$	$3{,}40 \cdot 10^7$	$2{,}10 \cdot 10^6$
3	$1{,}61 \cdot 10^9$	$2{,}29 \cdot 10^7$	$2{,}00 \cdot 10^7$	$6{,}10 \cdot 10^6$
4	$2{,}80 \cdot 10^9$	$3{,}22 \cdot 10^7$	$2{,}38 \cdot 10^8$	$3{,}20 \cdot 10^7$
5	$4{,}49 \cdot 10^9$	$1{,}70 \cdot 10^8$	$3{,}38 \cdot 10^7$	$3{,}36 \cdot 10^8$
6			$2{,}10 \cdot 10^7$	$3{,}68 \cdot 10^8$
7			$6{,}90 \cdot 10^8$	$3{,}92 \cdot 10^8$
8	$2{,}64 \cdot 10^9$	$9{,}04 \cdot 10^7$	$3{,}10 \cdot 10^7$	$3{,}40 \cdot 10^6$
9			$1{,}11 \cdot 10^8$	$4{,}30 \cdot 10^6$
10			$2{,}80 \cdot 10^8$	$4{,}52 \cdot 10^7$
11			$4{,}96 \cdot 10^8$	$8{,}60 \cdot 10^6$
12	$7{,}76 \cdot 10^9$	$6{,}18 \cdot 10^8$	$1{,}07 \cdot 10^8$	$1{,}40 \cdot 10^7$
13			$6{,}00 \cdot 10^8$	$1{,}30 \cdot 10^8$
14			$4{,}20 \cdot 10^8$	$7{,}60 \cdot 10^7$
15			$7{,}10 \cdot 10^8$	$8{,}60 \cdot 10^6$
16	$4{,}35 \cdot 10^9$	$3{,}02 \cdot 10^8$	$2{,}28 \cdot 10^9$	$3{,}20 \cdot 10^8$
17			$9{,}20 \cdot 10^8$	$7{,}70 \cdot 10^7$
18			$7{,}90 \cdot 10^8$	$1{,}06 \cdot 10^7$
19			$1{,}20 \cdot 10^9$	$2{,}56 \cdot 10^8$
20	$1{,}52 \cdot 10^9$	$6{,}96 \cdot 10^8$	$2{,}32 \cdot 10^8$	$3{,}20 \cdot 10^6$
21			$3{,}90 \cdot 10^7$	$3{,}30 \cdot 10^6$
22			$2{,}88 \cdot 10^8$	$5{,}30 \cdot 10^6$
23			$3{,}60 \cdot 10^7$	$4{,}40 \cdot 10^6$
24	$1{,}62 \cdot 10^9$	$4{,}29 \cdot 10^8$	$3{,}70 \cdot 10^7$	$3{,}90 \cdot 10^6$
25			$2{,}36 \cdot 10^8$	$1{,}06 \cdot 10^8$
26			$7{,}20 \cdot 10^7$	$2{,}06 \cdot 10^7$
27			$1{,}72 \cdot 10^8$	$8{,}00 \cdot 10^6$

Table 3.5. Mean values of absorbance and reducing sugars concentrations in pretreated starch before SSF process.

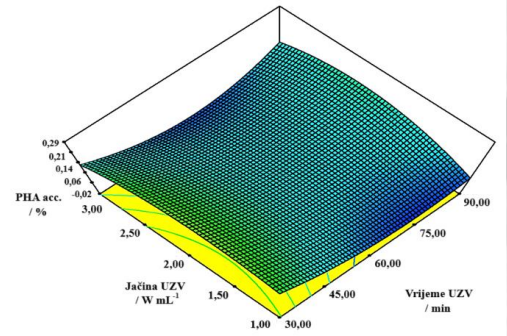
Exp.	ABS / -	c(reducing sugars) / mmol L^{-1}
1	0,004	0,170
2	0,004	0,168
3	0,003	0,164
4	0,004	0,168
5	0,004	0,168
6	0,001	0,151
7	0,002	0,155
8	0,002	0,159
9	0,004	0,166
10	0,002	0,157
11	0,002	0,157
12	0,004	0,170
13	0,001	0,153
14	0,005	0,173
15	0,005	0,173
16	0,003	0,162
17	0,003	0,164
18	0,019	0,250
19	0,005	0,172
20	0,004	0,170
21	0,003	0,164
22	0,003	0,162
23	0,005	0,175
24	0,005	0,175
25	0,009	0,194
26	0,007	0,183
27	0,011	0,205

3.3. Waste starch pretreatment optimisation

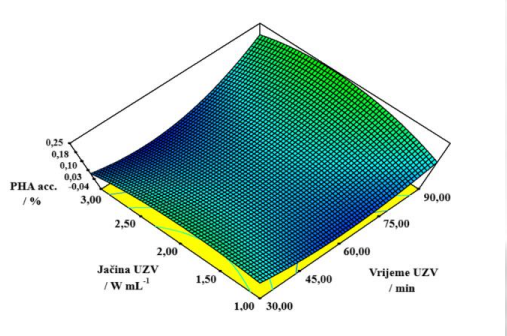
Waste starch pretreatment optimisation for PHA production



a)



b)

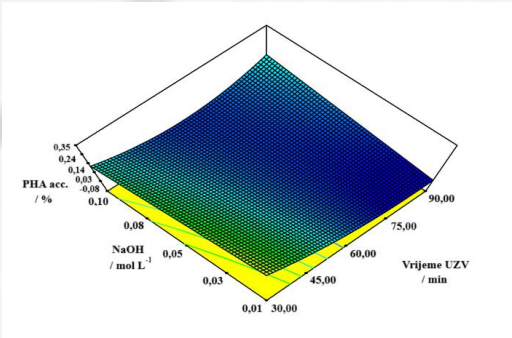


c)

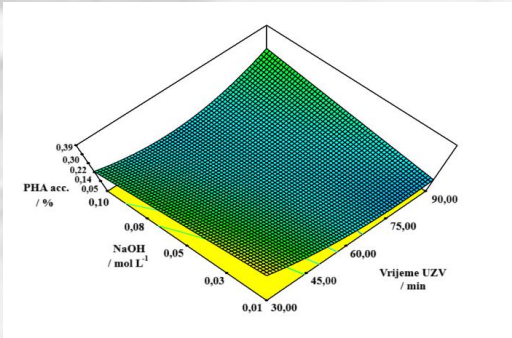
Response surfaces for PHA accumulation in correlation with US power and pretreatment time (constant values of NaOH concentrations a) 0,01 mol L^{-1}; b) 0,05 mol L^{-1}; c) 0,1 mol L^{-1}).

Table 3.6. Optimal conditions for starch pretreatment resulting with the highest PHA accumulation value.

Time / min	US power / W mL^{-1}	*c*(NaOH) / mol L^{-1}
30,00	1,71	0,01



a)



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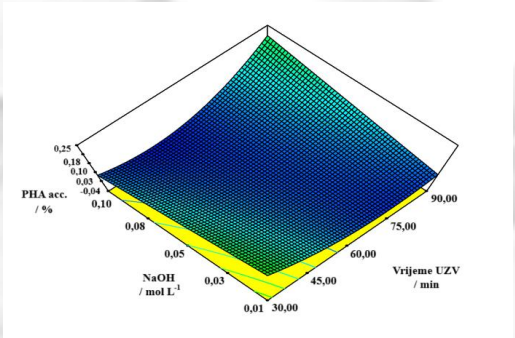


c)

Response surfaces for PHA accumulation in correlation with NaOH concentration and pretreatment time (constant values of US power a) 1 W mL^{-1}; b) 2 W mL^{-1}; c) 3 W mL^{-1}).

Table 3.7. Statistical data obtained with ANOVA analysis.

Standard deviation	0,042
Median	0,11
C. V. %	37,86
PRESS	0,073
R^2	0,9132
Adjusted R^2	0,8812
Estimated R^2	0,8131
Adequate precision	19,648

3.3. Waste starch pretreatment optimisation

Pretreated starch characterisation after SSF process

Table 3.8. Pretreated starch characterisation (before SSF process).

Exp.	$w(H_2O)_{sr.}$ / %	$w(DM)_{sr.}$ / %	$w(VM)_{sr.}$ / %	pH	κ / mS cm^{-1}	$\gamma(O_2)$ / mg L^{-1}	T / °C
1	14,60	85,40	99,65	10,214	0,457	8,30	21,8
2	14,42	85,58	98,74	10,625	2,910	8,29	21,8
3	19,30	80,70	97,79	10,650	2,950	7,30	21,8
4	14,53	85,47	99,68	9,717	0,430	4,58	21,8
5	18,00	82,00	98,85	10,553	2,350	8,35	22,4
6	35,99	64,01	98,23	10,662	3,030	8,32	22,4
7	19,12	80,88	99,50	10,022	0,496	7,70	22,3
8	43,13	56,87	99,05	10,390	2,320	7,97	23,0
9	58,47	41,53	98,78	10,677	5,600	7,98	23,0
10	16,72	83,28	99,56	9,919	0,424	6,50	23,3
11	18,08	81,92	98,66	10,109	1,963	1,78	23,3
12	26,28	73,72	97,92	10,540	3,280	5,96	23,3
13	17,72	82,28	99,57	8,851	0,444	2,22	23,2
14	17,65	82,35	98,71	10,522	2,280	7,91	23,3
15	64,56	35,44	97,66	10,454	2,970	6,20	23,4
16	16,68	83,32	99,68	5,633	0,411	3,30	22,8
17	18,27	81,73	98,78	9,359	1,091	3,78	22,6
18	72,45	27,55	98,95	7,495	2,240	4,28	22,8
19	17,48	82,52	99,61	6,075	0,353	3,40	22,7
20	17,69	82,31	98,66	9,866	1,138	6,10	22,9
21	10,24	89,76	97,52	10,271	2,430	7,27	22,7
22	16,41	83,59	99,63	7,267	0,324	1,74	22,8
23	17,29	82,71	98,78	9,841	1,264	6,24	22,8
24	76,12	23,88	98,59	10,314	3,980	6,02	24,0
25	15,97	84,03	99,66	9,457	0,247	6,12	24,2
26	16,53	83,47	98,75	9,771	1,233	7,01	23,8
27	13,01	86,99	96,66	9,807	3,160	5,53	24,1

Table 4.9. CFU values after 7 days of SSF process.

	Suspension + waste (final)	
	HA	**SA**
Exp.	CFU / cells g_{DM}^{-1}	CFU / cells g_{DM}^{-1}
1	$8{,}16 \cdot 10^{7}$	$1{,}84 \cdot 10^{7}$
2	$5{,}73 \cdot 10^{7}$	$1{,}04 \cdot 10^{8}$
3	$3{,}30 \cdot 10^{7}$	$3{,}95 \cdot 10^{7}$
4	$2{,}32 \cdot 10^{9}$	$3{,}60 \cdot 10^{8}$
5	$3{,}70 \cdot 10^{7}$	$2{,}80 \cdot 10^{7}$
6	$3{,}10 \cdot 10^{7}$	$3{,}36 \cdot 10^{7}$
7	$3{,}00 \cdot 10^{7}$	$5{,}90 \cdot 10^{6}$
8	$4{,}00 \cdot 10^{7}$	$3{,}40 \cdot 10^{6}$
9	$4{,}70 \cdot 10^{7}$	$3{,}30 \cdot 10^{6}$
10	$9{,}80 \cdot 10^{7}$	$3{,}70 \cdot 10^{6}$
11	$2{,}88 \cdot 10^{8}$	$9{,}60 \cdot 10^{6}$
12	$3{,}10 \cdot 10^{7}$	$5{,}50 \cdot 10^{6}$
13	$2{,}64 \cdot 10^{8}$	$4{,}00 \cdot 10^{7}$
14	$1{,}06 \cdot 10^{8}$	$2{,}56 \cdot 10^{7}$
15	$3{,}10 \cdot 10^{7}$	$3{,}30 \cdot 10^{6}$
16	$6{,}90 \cdot 10^{8}$	$9{,}60 \cdot 10^{6}$
17	$3{,}10 \cdot 10^{7}$	$3{,}20 \cdot 10^{6}$
18	$5{,}00 \cdot 10^{8}$	$3{,}00 \cdot 10^{6}$
19	$1{,}40 \cdot 10^{9}$	$6{,}80 \cdot 10^{7}$
20	$7{,}10 \cdot 10^{7}$	$4{,}20 \cdot 10^{6}$
21	$1{,}28 \cdot 10^{8}$	$3{,}50 \cdot 10^{6}$
22	$4{,}53 \cdot 10^{8}$	$1{,}09 \cdot 10^{8}$
23	$3{,}10 \cdot 10^{7}$	$4{,}00 \cdot 10^{6}$
24	$3{,}90 \cdot 10^{8}$	$5{,}00 \cdot 10^{7}$
25	$3{,}00 \cdot 10^{8}$	$6{,}50 \cdot 10^{6}$
26	$3{,}90 \cdot 10^{8}$	$3{,}20 \cdot 10^{7}$
27	$8{,}90 \cdot 10^{7}$	$3{,}30 \cdot 10^{6}$

Table 4.10. Mean values of absorbance and reducing sugars concentrations in pretreated starch after SSF process.

Exp.	ABS / -	c(reducing sugars) / mmol L^{-1}
1	0,003	0,162
2	0,005	0,172
3	0,003	0,162
4	0,024	0,278
5	0,003	0,162
6	0,001	0,150
7	0,010	0,201
8	0,003	0,164
9	0,001	0,151
10	0,000	0,148
11	0,001	0,153
12	0,003	0,164
13	0,006	0,177
14	0,147	0,955
15	0,003	0,162
16	0,014	0,223
17	0,014	0,223
18	0,011	0,206
19	0,011	0,206
20	0,016	0,234
21	0,013	0,219
22	0,009	0,197
23	0,016	0,232
24	0,001	0,151
25	0,011	0,205
26	0,010	0,201
27	0,277	1,670

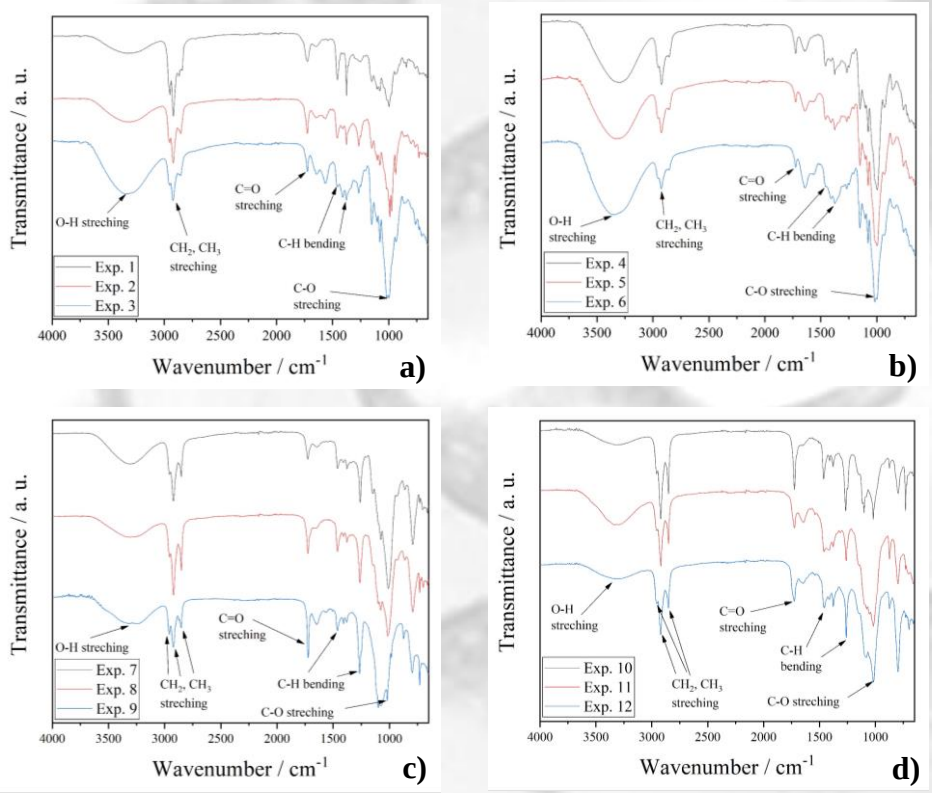
3.4. PHA extraction and purification

Table 3.11. Residual biomass and PHA accumulation values obtained after 7 days of SSF process.

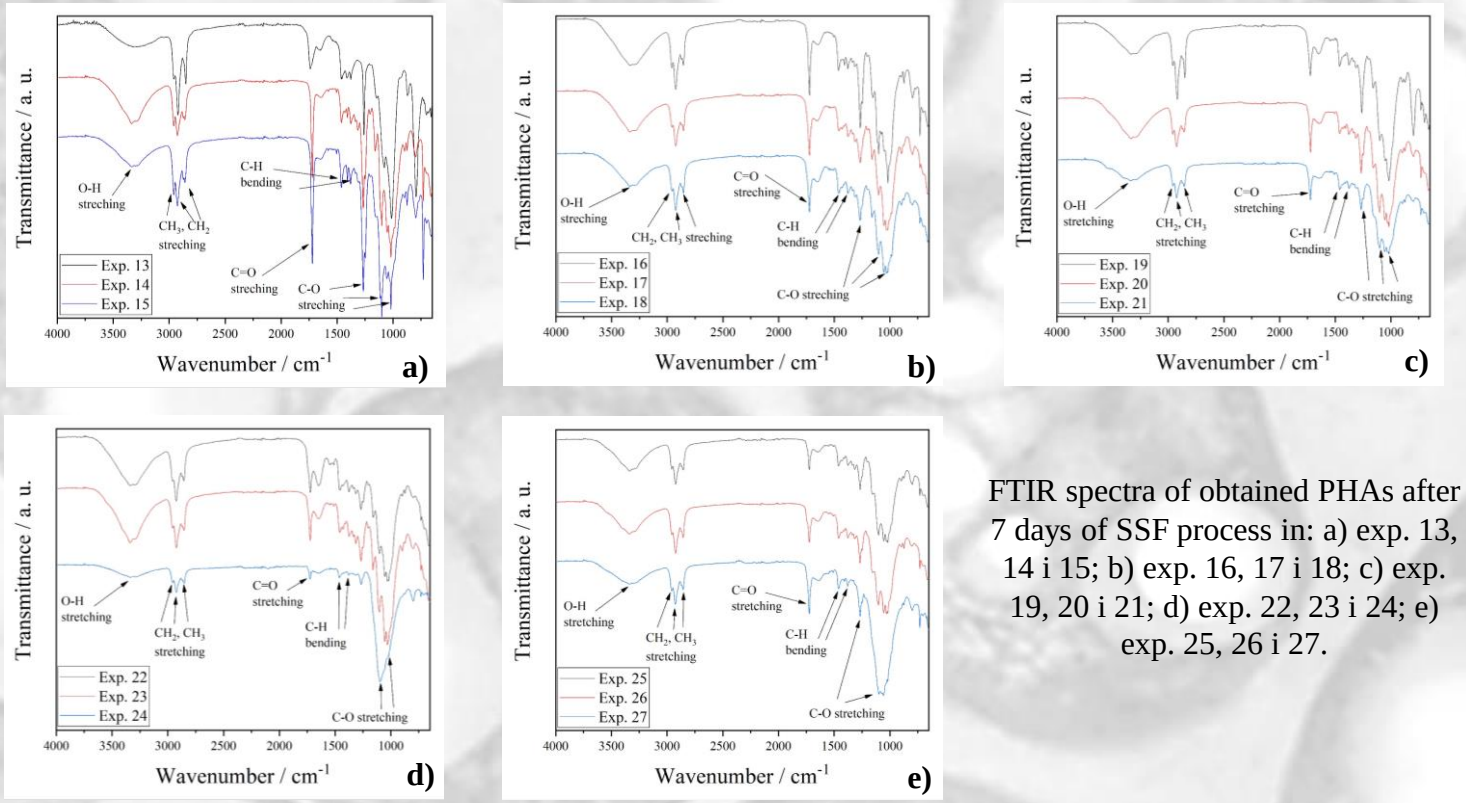
Exp.	Residual biomass/ g L^{-1}	PHA accumulation / %
1	78,6510	0,0670
2	103,4366	0,3184
3	88,2315	0,1498
4	86,6649	0,5572
5	81,5187	0,1872
6	87,7520	0,2145
7	114,5493	0,0063
8	86,1329	0,0582
9	88,5636	0,0278
10	86,3361	0,0311
11	85,8451	0,0033
12	86,4187	0,0023
13	75,4795	0,0069
14	87,0705	0,0256
15	89,5635	0,0684
16	104,4491	0,0274
17	109,1973	0,0098
18	101,4261	0,0060
19	98,7314	0,0212
20	90,1461	0,1347
21	88,8875	0,0404
22	88,2411	0,0388
23	90,2490	0,0308
24	93,6751	0,1223
25	89,6794	0,0333
26	90,4475	0,0302
27	93,5773	0,5105

3.5. PHA characterisation

FTIR



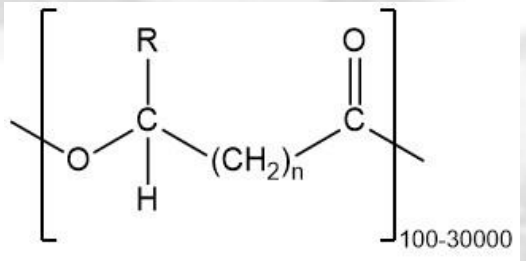
FTIR spectra of obtained PHAs after 7 days of SSF process in:
a) exp. 1, 2 i 3; b) exp. 4, 5 i 6; c) exp. 7, 8 i 9; d) exp. 10, 11 i 12.



FTIR spectra of obtained PHAs after 7 days of SSF process in: a) exp. 13, 14 i 15; b) exp. 16, 17 i 18; c) exp. 19, 20 i 21; d) exp. 22, 23 i 24; e) exp. 25, 26 i 27.

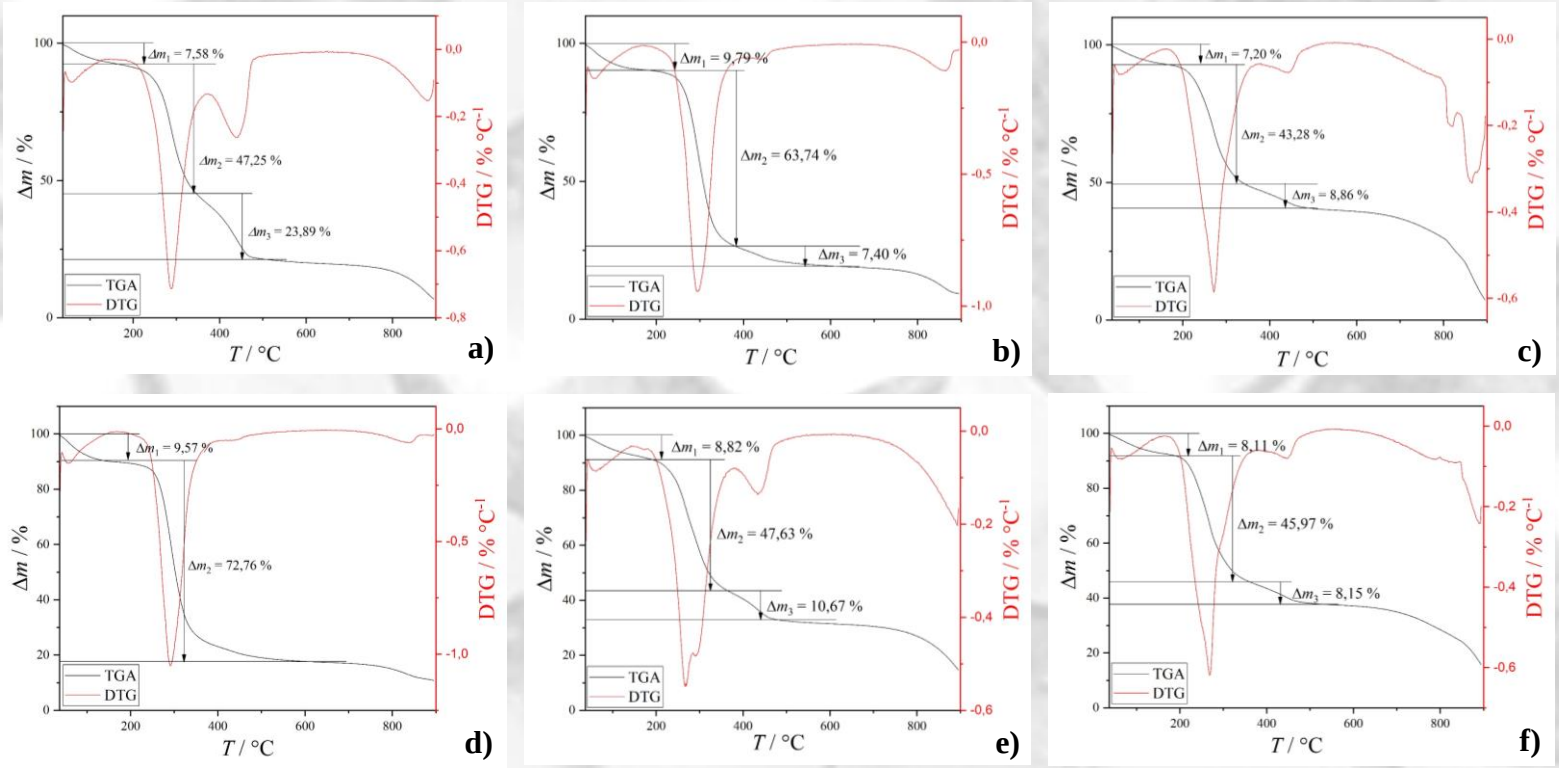
Tablica 5.2. Wavenumber values for specific peaks in infrared spectra for PHA.

Bond structure	Resonance type	Wavenumber / cm^{-1}
O – H	Stretching	3200 – 3550
CH_2, CH_3	Stretching	2850 – 3000
C = O	Stretching	1715 – 1730
C – H	Bending	1350 – 1500
C – O	Stretching	990 – 1300



3.5. PHA characterisation

TGA



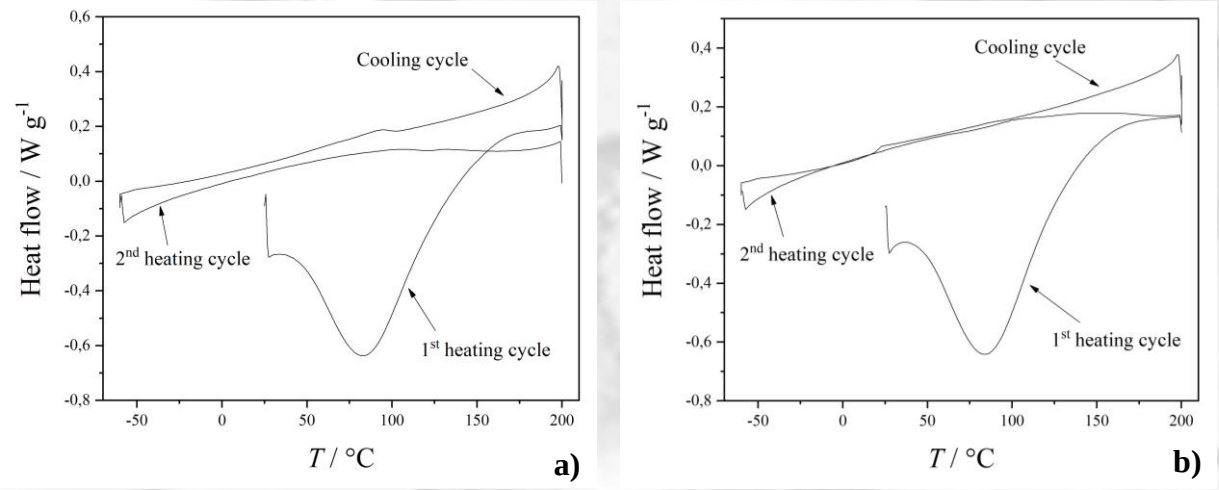
Thermograms of PHA obtained after 7 days of SSF process in experiments: a) 1; b) 2; c) 3; d) 4; e) 5; f) 6.

Tablica 3.13. Value of specific mass alterations, temperatures measured at the beginning and at the end of PHA degradation process, temperatures measured at the highest speed of PHA degradation and inorganic residues for the first 6 experiments.

Exp.	Δm_1 / %	Δm_2 / %	Δm_3 / %	$T_{poč.}$ / °C	$T_{kon.}$ / °C	T_1(max.) / °C	T_2(mSax.) / °C	T_3(max.) / °C	Residue / %
1	7,58	47,25	23,89	164,24	510,67	59,14	287,07	439,22	6,44
2	9,79	64,74	7,40	167,57	587,03	59,90	294,14	449,05	9,11
3	7,20	43,28	8,86	165,59	552,97	59,70	273,27	441,37	7,34
4	9,57	72,76	/	161,82	596,46	59,65	292,13	/	10,79
5	8,82	47,63	10,67	180,96	559,28	59,31	268,35	435,66	14,96
6	8,11	45,97	8,15	182,89	526,97	59,26	270,29	450,99	15,87

3.5. PHA characterisation

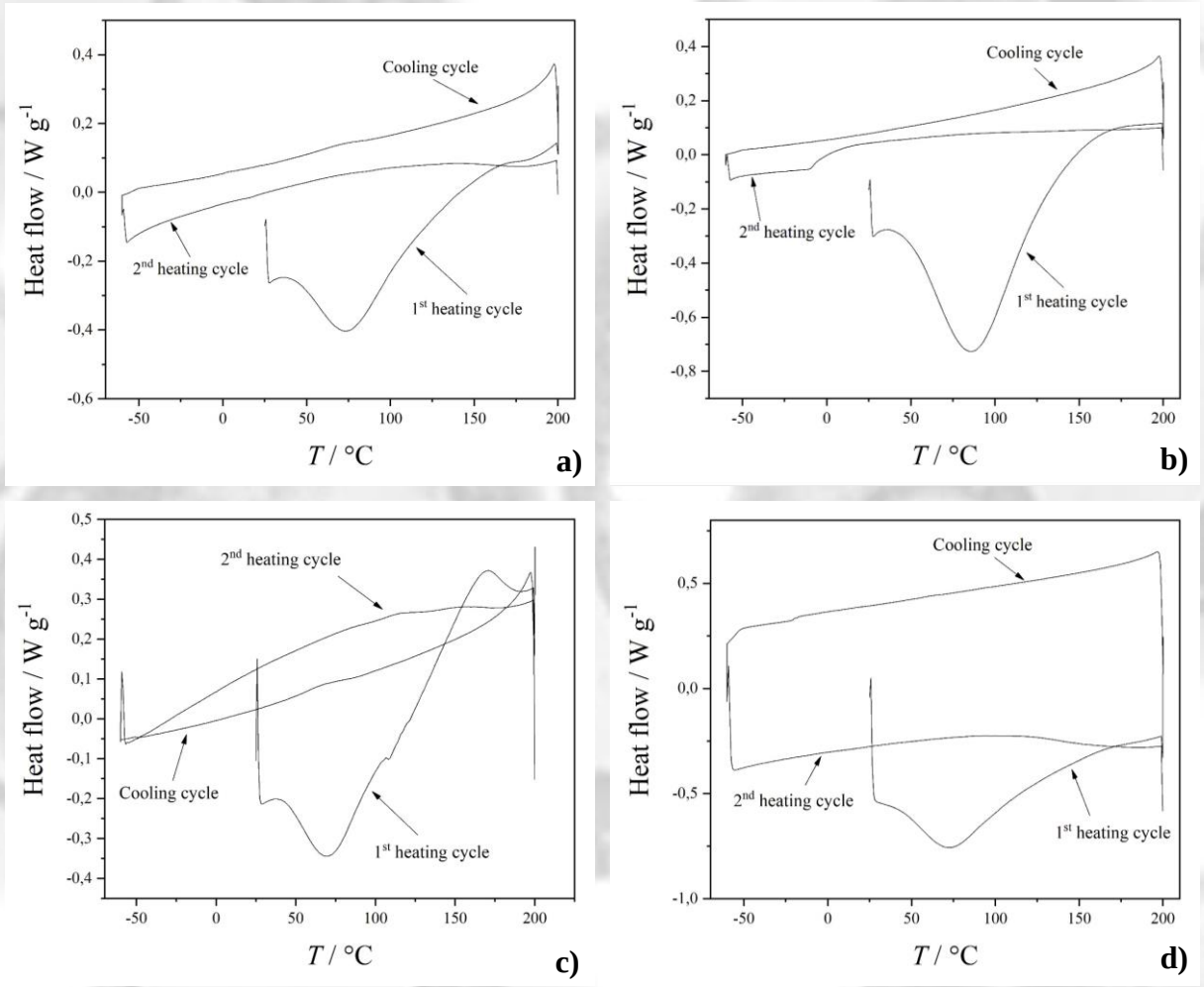
DSC



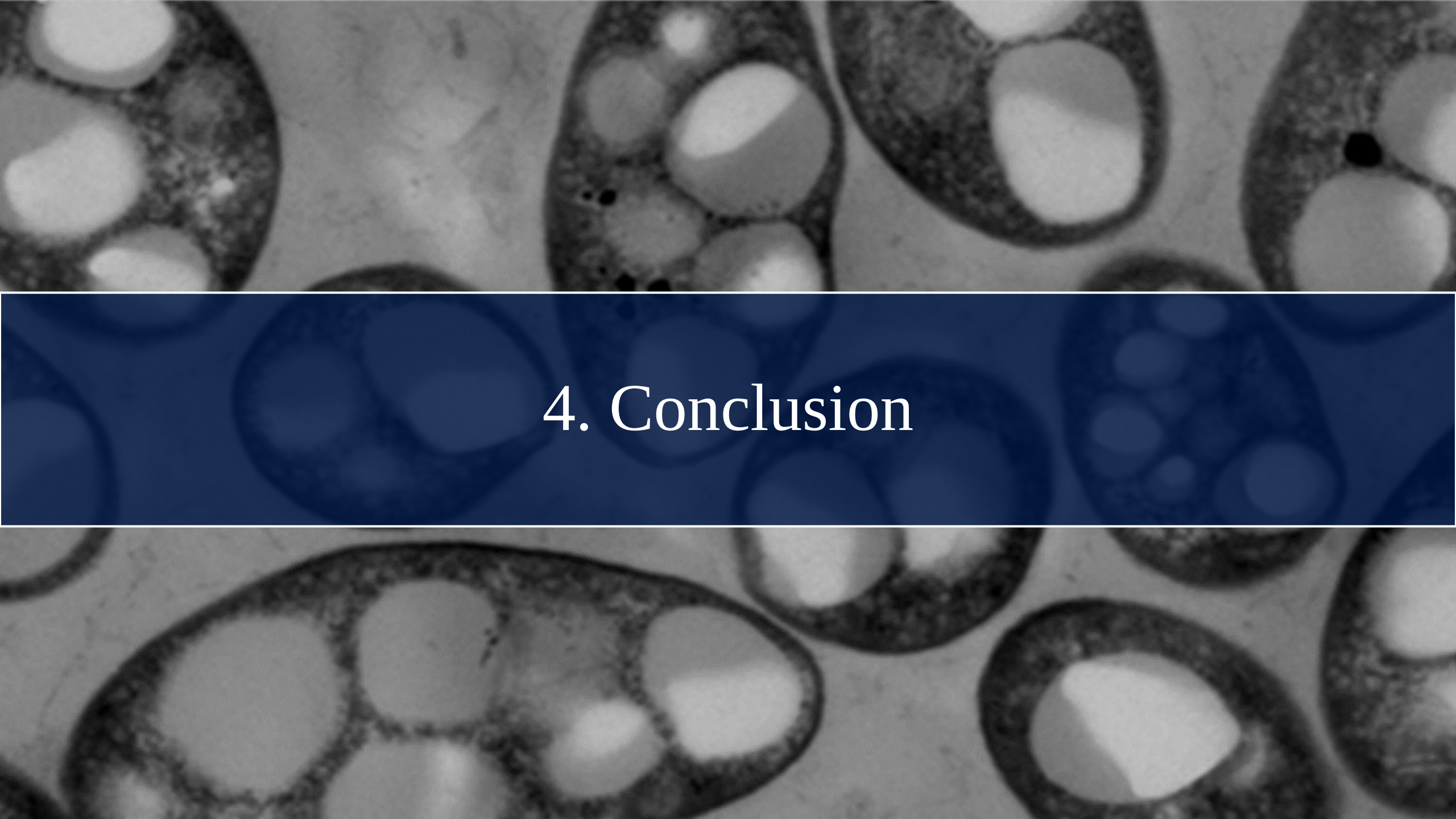
DSC curves for PHA obtained after 7 days of SSF fermentations in experiments: a) 1; b) 2.

Tablica 3.14. Values of melting temperature, T_m, glass transition temperature, T_g and melting entalpy ΔH_m for PHA obtained in first 6 experiments.

Exp.	T_g / °C	T_m / °C	ΔH_m / J g^{-1}
1	/	95,47	6,71
2	22,95	100,26	6,84
3	3,67	84,30	10,99
4	-10,05	/	/
5	/	115,30	4,19
6	-21,04	64,13	0,00



DSC curves for PHA obtained after 7 days of SSF fermentations in experiments: a) 3; b) 4; c) 5; d) 6.



4. Conclusion

Based on the scientific research of optimisation of PHA production from waste starch, the results have given the following conclusions:

- Combination of ultrasound and alkaline solution as a pretreatment method for waste starch has shown to be too intense, due to the possibility of ireverse starch gelatinisation occuring.
- The loss of moisture in starch structure after the pretreatment is a result of starch retrogradation, while increase in moisture occurs as a product of methabolic processes in microorganism cells.
- Starch contains low levels of reducing sugars due to polymer nature of starch, as well as due to technical reasons while coducting the experiments.
- Highest PHA accumulation was obtained in experiment 4 (0,5572 %, after 30 min of pretreatment, with the ultrasound power of 2 W mL^{-1} and NaOH concentration of 0,01 mol L^{-1}), which correlates with the given experiment design.
- Low PHA accumulations are the result of low reducing sugar content, starch pretreatment method, higher pH value and using mixed culture for SSF process. vrijednosti, to je teže rukovati sa škrobom i manje su vrijednosti akumulacije PHA.
- Low oxygen saturation after the conducted SSF process is a result of microorganisms using oxygen for the fermentation process.
- Lower CFU value after the SSF process is due to higher pH values of the supstrate.
- FTIR analysis showed the presence of PHA in the final product, which is proven by the peaks in FTIR spectra for C–O, O–H and C=O bonds stretching, as well as $–CH_3$, –CH2– bonds bending and stretching in PHA molecules.
- TGA i DSC analyses confirmed the possible presence of homopolymers poly(hydroxybutyrate) (PHB) and poly(hydroxyvalerate) (PHV), copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) with different 3HB and 3HV fractions and different scl-PHA and mcl-PHA, such as PHB, PHV and poly(hydroxyhexanoate) (PHHx).
- FTIR, TGA and DSC analyses aren’t completelly suitable for PHA identification; ^{1}H NMR ili GC analyses are mandatory.

Thank you!