Sustainable Production: Recycling of Bacterial Biomass Resulting from a Fermentation Process with *Klebsiella planticola*

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Dedicated to Prof. Dr. Đurđa Vasić-Rački on occasion of her 60th birthday

The Gram-negative bacterium *Klebsiella planticola* represents an interesting host organism for the production of biotechnological products. Microbial processes – especially those of aerobic cultivation – lead to the generation of considerable amounts of biomass, thus lowering the product yield. These are the reasons for studying methods for the recycling of biomass from *K. planticola*. It will be shown that it is possible to disintegrate the microbial biomass – preferably by means of high pressure homogenization followed by a protease treatment of the resulting slurry of debris – in an efficient way and to recycle at least the soluble part as cultivation medium component. A recycling yield of about 77 % can be achieved. By studying the growth of *K. planticola* in a cyclic batch cultivation strategy with maximal recycling of the biomass no adverse effects have been observed for a series of more than 9 consecutive cultivation cycles.

Key words:
Biomass recycling, microbial biomass, lysis, *Klebsiella planticola*, sustainable production, cyclic batch operation

Introduction

During the production of biotechnological bulk products biomass occurs as waste if biomass is not the product. Current methods for the disposal of biomass as waste are the degradation in sewage treatment plants, its use in animal feed as well as in landfilling and composting. This report deals with biomass recycling as a source of nutrients used in a single process. The strategy of biomass recycling is based on previous results obtained with different bacterial processes.[1–5]

The purpose of this work was the examination and development of methods for the recycling of biomass waste from the Gram-negative bacterium *Klebsiella planticola* as a media component in the fermentation process. After suitable treatment the biomass should be returned into the process of which it originated. Due to industrial applications in mind only methods were taken into consideration which would allow a transfer into industrial scale. A detailed comparison of using either yeast extract or *Klebsiella planticola* lysates will be given.

In terms of sustainable production the recycling of a large fraction of the bacterial biomass should save energy and material resources. In addition, the recycling of matter could lead to an improved efficiency of the production process due to the reduction of required raw materials and the decrease of the amount of waste.

Klebsiella planticola has successfully been used by the authors for expression and secretion of recombinant proteins for years.[6] Furthermore, the organism has been classified by German public authorities, and the US-American Food and Drug Administration (FDA) in the GRAS (generally recognized as safe) status.

Material and methods

Organisms and culture media

The strain *Klebsiella planticola* DSMZ Nr. 4617 (ATCC 15050) was used throughout these studies.

Batch cultivations for the production of bacterial biomass were carried out in complex Terrific Broth medium (TB), the composition of which is gathered in Table 1. Growth experiments were car-

Table 1 – *Composition of TB-medium*

Medium component	Mass concentration γ / g dm^{-3}
soy peptone	12.0
yeast extract	24.0
glycerol	4.0
KH_2PO_4	2.3
K_2HPO_4	11.5

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Table 2 – *Composition of the semi-synthetic medium*[7]

Medium component	Mass concentration γ / g dm^{-3}
glucose	40.00
yeast extract	2.00
$(NH_4)_2SO_4$	6.00
KH_2PO_4	2.00
$MgSO_4 \cdot 7\ H_2O$	0.40
$FeSO_4 \cdot 7\ H_2O$	0.02
$MnSO_4 \cdot H_2O$	0.02
maltose	10.00

ried out on semi-synthetic media as summarized in Table 2 with glucose and maltose as carbon sources.[7] Control of pH was accomplished in the first mentioned case with 2 mol dm^{-3} sodium hydroxide solution and w = 10 % phosphoric acid, and in the second case with w = 20 % sulphuric acid and w = 25 % ammonia solution. Yeast extract was acquired from Ohly (Type: KAT, Charge: EO100504) and soy peptone from UD Chemie (Type: CM, Charge: 700/203).

The yeast extract of the semi-synthetic medium (γ = 2 g dm^{-3}) was replaced by the *Klebsiella planticola* lysate. Therefore, the whole biomass obtained from one fermentation was lysed and after clarification the soluble part was used for substituting the yeast extract.

Cultivation conditions

Cultivations were carried out in a MBR bioreactor (MBR BIO REACTOR, Switzerland) with a total volume of 7 dm^3 operated with 5 dm^3 at an operating temperature of 37 °C and a pH of 7.0. The flow rate of air was fixed at Q = 5 dm^3 min^{-1}. The oxygen concentration was controlled by increasing the stirrer speed in the range of n = 500 to 2000 min^{-1}. The bioreactor was inoculated with 50 cm^3 of an overnight shaking flask culture, grown in the same medium. Cells were harvested by centrifugation for 10 min at 9.600 g. The lysis was either performed immediately or the cells were frozen and stored at –20 °C before lysis.

Lysis

After thawing the cells were suspended in tap water. During the freeze-thawing process a partial lysis of cells may have occurred. Lysis experiments were accomplished in 1,000 cm^3 shaking flasks closed with aluminium caps to prevent evaporation. The filling volume was about 300 cm^3. Biomass concentrations in the range of γ_x = 34 g dm^{-3} were used. Adjustment of pH was carried out with 2 mol dm^{-3} sodium hydroxide solution and 2 mol dm^{-3} hydrochloric acid. The degree of lysis was defined as:

$$Y = 1 - \frac{\gamma_X}{\gamma_{X0}} \quad (1)$$

with γ_X the mass concentration of biomass or solids at time t, and γ_{X0} the same at the time when lysis was started.

For enzymatic lysis an industrial serine protease (Alcalase® 2.4 L FG, Novozymes, Denmark) with an activity of 2.4 AU g^{-1} (AU = Anson Units) was used. The enzymatic lysis was performed at temperature optimum (55 °C) and pH optimum (pH 8.5) of this protease.

For high pressure homogenisation a lab homogeniser Mini-Lab 8.30 H (Rannie) was used. A disintegration pressure of 600 bar and a number of 3 passages were experimentally established for *K. planticola*. The temperature was kept below 30 °C.

For the production of lysate for growth experiments the frozen cell pellet was resuspended in 2 dm^3 tap water and applied to the disintegration procedure. Then the suspension was centrifuged for 30 min at 17.000 g, decanted, and either used immediately or stored at –20 °C.

Analyses

During cultivations with semi-synthetic media samples were taken at one hour intervals to determine the optical density at a wavelength of 600 nm (Photometer UV-1202, Shimadzu), the glucose mass concentration (Enzytec™ D-Glucose, Scil Diagnostics), the maltose mass concentration from the mass concentration of reducing sugars,[8] the acetate concentration by HPLC (Macherey-Nagel, Nucleogel ION 300 OA column Art. Nr. 719501) and the mass concentration of dry biomass by gravimetry.

The yield or degree of lysis was monitored by measuring the dissolved protein concentration in the supernatant after clearing the lysate. Protein mass concentration was estimated by means of the Bradford method (Roti-Nanoquant™, Roth) with bovine serum albumin as standard.

The quality of the lysates was assessed by analysing the content of total carbon and total nitrogen (Vario EL, Elementar), protein (see above), amino acids by HPLC,[9] deoxyribonucleic acid according to Dische[10] and acetate (see above).

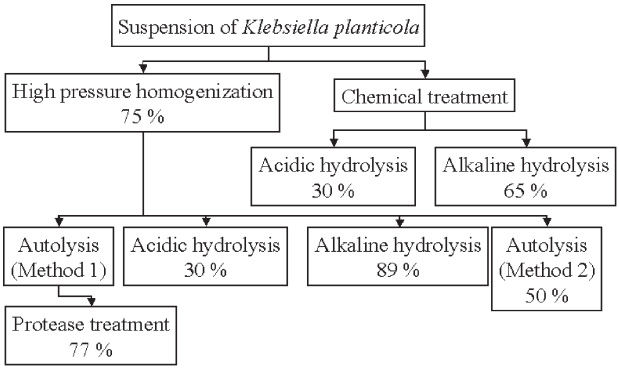
Results and discussion

Design of experiments

Biomass of *Klebsiella planticola* for lysis experiments was obtained by cultivations on complex Terrific Broth media. After the harvest of cells, different methods of lysis were evaluated in shake flask experiments. For the following cultivation experiments biomass was produced on semi-synthetic media. To compare the influence of the lysate, cultivations on semi-synthetic media with and without lysate were carried out. In repetitive growth experiments, finally, yeast extract was replaced by lysate. The biomass obtained was disintegrated and reapplied as media component for starting the following cultivation cycle.

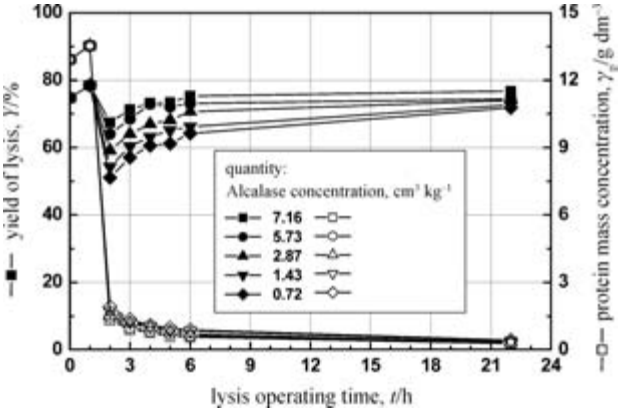
Lysis experiments

The main objective was to find the most cost efficient option for the lysis of *Klebsiella planticola* cells. Lysis was carried out by means of high pressure homogenisation, autolysis, enzymatic treatment, chemical hydrolysis, and several combinations of these methods. The influence of different operating variables like enzyme concentration, temperature, pH, as well as, in case of high pressure homogenisation, the number of passages and the disintegration pressure was examined. Fig. 1 shows an overview of the accomplished lysis experiments in shake flask scale. The maximal degrees of lysis obtained are shown in addition. A decrease in the degree of lysis could be observed during lyses experiments due to the precipitation of mainly proteins after raising the temperature.



F i g . 1 – *Lysis of Klebsiella planticola. The maximal degree of lysis is given for each method*

The results of an enzymatic lysis with prior high pressure homogenisation followed by a short phase of autolysis are presented at first. Preliminary experiments showed that a disintegration pressure of $P = 600$ bar and a number of three passages were optimal. Fig. 2 shows the dependence of the protein



F i g . 2 – *High pressure homogenisation of Klebsiella planticola combined with enzymatic lysis under variation of enzyme mass concentration. The enzyme solution was added after one hour of autolysis.*

concentration and the degree of lysis as a function of operating time for a series of different Alcalase concentrations. After high pressure homogenisation the degree of lysis was about 75 % ($t = 0$ h). During the following autolysis a low increase in protein concentration and in the degree of lysis could be observed. Obviously, the liberated intracellular enzymes e. g. proteases and nucleases were able to degrade insoluble substances. This autolysis was accomplished at a pH of 8.5 and a temperature of 37 °C.

As a result of raising the temperature to 55 °C after autolysis (Method 1), and adding enzyme solution, a decrease of the concentration of soluble protein and of the degree of lysis occurred as shown in Fig. 2. The lower the enzyme mass concentration the bigger was this effect. The soluble as well as the solid proteins were degraded by the Alcalase which may be deduced from the fact that the degrees of lysis rose to their final values in the range of 72 to 77 %. Higher enzyme mass concentrations merely resulted in a faster lysis, but the effect was of minor extent. It could be shown that an Alcalase concentration lower than 0.001 $dm^3\ kg^{-1}$ referred to cell dry mass was sufficient to reduce the protein concentration in the cell lysates very efficiently.

Analyses of the lysates

Only results of lysates obtained by high pressure homogenisation combined with Alcalase treatment will be discussed since growth experiments were accomplished with these lysates.

The total carbon and total nitrogen content of *K. planticola* lysates were found to be in the range of 36.4–41.4 % and 9.3–10.8 %, respectively. The corresponding values for the yeast extract (Producer: Ohly, type: KAT, Charge: EO100504) were 40.5 % and 12.4 %, respectively. Protein mass con-

centrations of 0.36-0.70 g dm^{-3} were observed. A 12 % SDS-PAGE showed that the molecular mass of the proteins were in the range of 10 and 40 kDa. Corresponding analyses of yeast extract showed no detectable concentration of proteins at all. Thus, the degree of hydrolysis of the proteins seemed to be higher in the yeast extract than in *Klebsiella* lysates. This conclusion may be drawn, because the Bradford test only detected proteins with a molecular mass higher than 3 kDa.[11] The acetate mass concentration in the lysates was about 1.4-2.3 g L^{-1}. Acetate could not be detected in yeast extract.

Table 3 shows the amino acid spectrum found in these lysates. The mass fraction of total amino acids in the lysates was between w = 31 and 41 % compared with that in yeast extract of 35 %. This corresponded to a total solids mass concentration in the lysates of 25.4 g dm^{-3} to 35.1 g dm^{-3}. The degree of lysis reached values of up to 77 %. Mass fractions of DNA of w = 2.2-3.4 % were found in the lysates, whereas yeast extract contained 0.77 %.

Table 3 – *Mass fraction of amino acids in the lysates and yeast extract (in brackets)*

Amino acid	Mass fraction, w / %	Amino acid	Mass fraction, w / %
aspartic acid	2.6 – 3.3 (2.4)	tyrosine	1.6 – 2.1 (0.6)
glutamic acid	3.0 – 4.6 (4.6)	lysine	1.6 – 2.2 (2.5)
asparagine	0.6 – 1.7 (1.5)	methionine	1.1 – 1.4 (0.8)
serine	0.7 – 1.9 (2.1)	valine	2.3 – 2.8 (2.5)
glutamine	0.2 – 0.8 (0.4)	tryptophan	0.6 – 0.7 (0.6)
histidine	0.4 – 0.9 (0.5)	phenylalanine	1.6 – 2.1 (2.1)
glycine	1.8 – 2.5 (1.3)	isoleucine	2.0 – 2.9 (1.9)
threonine	1.7 – 2.5 (2.0)	leucine	3.7 – 5.1 (4.1)
arginine	2.2 – 3.4 (1.7)	alanine	2.4 – 3.6 (3.4)

Cultivations on semi-synthetic media

Fermentations with the semi-synthetic medium were carried out in order to produce biomass and to obtain information about the growth performance of the bacterial strain. Results from five cultivations were collected.

Cultivations of *Klebsiella planticola* on the semi-synthetic medium showed three distinct growth phases. This could be clearly seen from the carbon dioxide concentration in the outlet air as shown in Fig. 3. In the first growth phase (0-5 h), in which the amino acids of the yeast extract were utilized, the specific growth rate could be determined with 0.92-1.14 h^{-1}. In the following glucose metabolizing phase (5-10 h) the specific growth rate was between 0.62 and 0.68 h^{-1}. In the third phase (10-12.5 h) obviously maltose was consumed at a much lower specific growth rate. The maltose concentration was determined from the concentration of reducing sugars still showing a signal after glucose was entirely consumed. Maximal optical densities of 44-49 corresponding to a biomass concentration of 19.5-20.1 g dm^{-3} based on dry matter could be achieved.

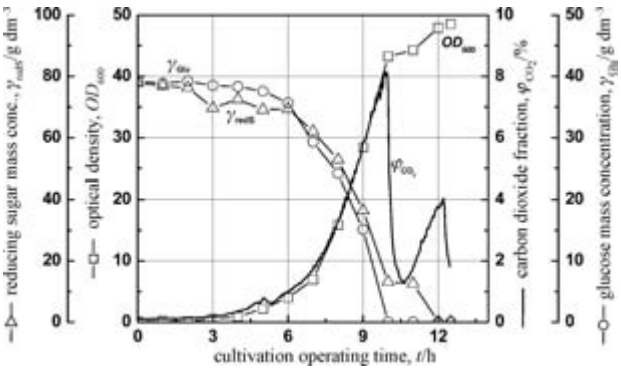
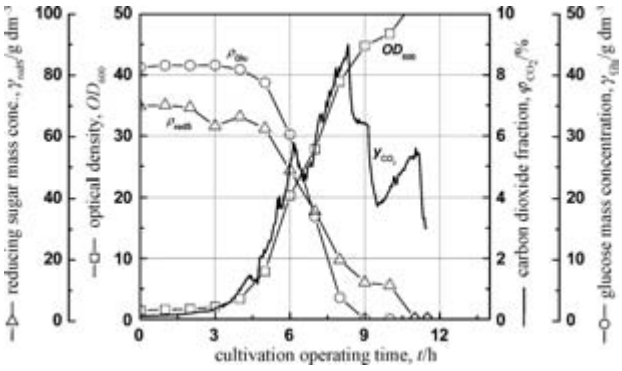


Fig. 3 – *Batch cultivation of Klebsiella planticola on the original semi-synthetic medium (without lysate)*

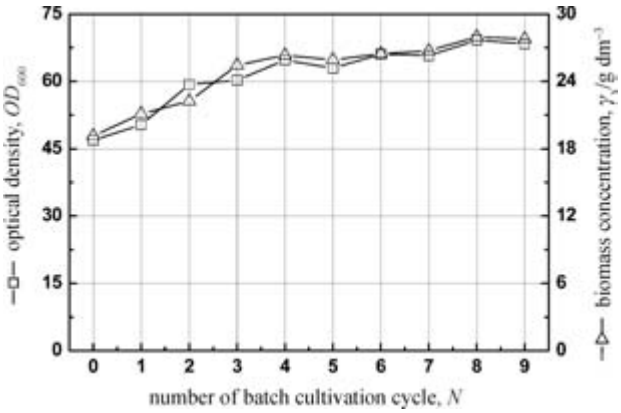
The total carbon balance shows that at the end of the cultivation 41.5 % (40.3 g) of the initial carbon (97.3 g) was bound in the biomass, 39.4 % (38.1 g) was gone as carbon dioxide in the waste air, and 16.3 % (15.9 g) still remained in the medium. It could be determined that nearly 90 % of the remaining carbon in the medium was acetate. These results are average values of five fermentations. During the fermentation process ammonia solution was used for pH-control leading to a more complex nitrogen mass balance. This nitrogen source was important for avoiding limitations. However, accumulation of nitrogen in the medium could be observed.

Fig. 4 shows the cultivation on the semi-synthetic medium, but this time using lysate instead of yeast extract. The yeast extract was replaced by 51 g dried lysate obtained from processing the biomass from one of the previously introduced cultivations. At least three different growth phases were observed. In the first phase the amino acids of the lysate were metabolised, followed by glucose and finally maltose.

The carbon mass balance showed that 53.5 % of the carbon was bound in the biomass, 32.6 %



F i g . 4 – *Batch cultivation of Klebsiella planticola on the semi-synthetic medium in which yeast extract was replaced by lysate*



F i g . 5 – *Batch cultivation experiments with total recycling of biomass. The data shown are those obtained at the end of each cultivation cycle. The first cultivation (0) was carried out on original medium with yeast extract.*

was gone in the waste air, and 17.3 % still remained in the medium. It could be demonstrated that this carbon content in the medium was composed of acetate and nucleic acids which were liberated by cell disintegration during the production of the lysate. These nucleic acids could not be utilized by the cells, probably due to the lack of extracellular nucleases.

Recycling

The whole cell pellet of a fermentation was used for the preparation of a lysate, which would replace the yeast extract in the semi-synthetic medium in the next fermentation cycle. Only the soluble part was used for this purpose. Due to the recycling of the whole biomass the carbon concentration increased in comparison to the original medium. Table 4 shows the carbon masses in the cultivation cycles at the beginning of the cultivations. The total carbon mass includes the carbon of glucose, maltose, and in the case of the original medium in yeast extract or in the other cases in lysate. The total carbon mass increased from 91.7 g to 142.1 g – referring to a standard culture volume of 5 dm^{-3}.

Fig. 5 shows the maximal optical densities observed and the corresponding biomass concentrations referred to dry matter for the cultivations of *K. planticola* with biomass recycling. Both variables increased significantly during the first four cycles due to total biomass recycling. Due to the gentle lysis conditions important growth factors like vitamins and other cosubstrates seemed not to be destroyed.

The cultivation time could be reduced due to the fact that the specific growth rates were higher and in some cases the adaption phase was shorter compared with the cultivation in the presence of yeast extract (data not shown). Thus, recycling of biomass in the form of soluble lysates resulted in higher productivities with respect to biomass formation.

The carbon balance showed that 43.0-48.3 % was used for formation of biomass during the fermentation cycles. With increasing number of cycles the amount of residual carbon in the medium rose considerably as documented in Table 4. However, a constant value of about 25 % was observed after the forth cycle, showing that a kind of steady state might have been achieved with respect to biomass recycling.

T a b l e 4 – *Carbon masses at the beginning and at the end of the fermentation cycles*

Cycle number N	Initial mass of soluble carbon in the medium $m_{C,0}$ / g	Final mass of soluble carbon in the medium $m_{C,f}$ / g	Final fraction of residual carbon in the medium w / %
0	91.7	17.9	19.6
1	115.7	20.9	18.0
2	112.0	19.4	17.3
3	130.8	20.6	15.8
4	131.0	30.3	23.1
5	136.7	36.5	26.7
6	139.0	40.5	29.1
7	143.5	37.2	25.9
8	140.9	37.4	26.5
9	142.1	34.9	24.6

Conclusions

It could be shown that it is possible to disintegrate biomass from *Klebsiella planticola* cultivations in an efficient way and to recycle the soluble

part as medium component. The results confirm findings for other bacterial processes that recycling of bacterial biomass instead of using conventional media components like yeast extract should be feasible. In consequence, the amount of waste biomass could clearly be reduced.

The method developed for disintegration and lysis of the cells used high pressure homogenisation and protease (alcalase) treatment. High-pressure homogenisation is widely used for industrial cell disruption for the release of intracellular products. Therefore, this method is applicable on large scale. The use of a technical enzyme lead to a recycling yield of at least 95 % of the proteins liberated. The enzyme concentration required could be kept below 1 litre per ton of dry biomass.

At last, the recycling of approximately 77 % of the biomass shows that no disadvantages for growth and biomass formation could be observed for a series of more than 9 consecutive fermentation cycles.

ACKNOWLEDGEMENT

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Symbols

m – mass, g
n – stirrer speed, min^{-1}
Q – volume flow rate, $dm^3\ min^{-1}$
OD_{600} – optical density at λ = 600 nm, –
p – pressure, bar
t – time, h
w – mass fraction, %
φ – volume fraction, –
Y – yield of lysis; degree of lysis, –
λ – wavelength, nm
μ – specific growth rate, h^{-1}
γ – mass concentration, g dm^{-3}

Subscripts

C – carbon
CO_2 – carbon dioxide
f – final
Glu – glucose
P – protein
redS – reducing sugar
X – biomass; based on dry matter
o – initial

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