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Development of an integrated bioconversion process for the production of 1,3-propanediol from raw glycerol waters

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Abstract

The valorisation of glycerol produced in a constantly increasing amount as a by-product from fat processing becomes a serious problem for the related industry. The refining of this glycerol to pharmaceutical quality is energy-consuming and does not represent a cost-effective alternative, especially not for biodiesel producers. Another possibility for valorisation of raw glycerol is the microbiological conversion into the important intermediate chemical 1,3-propanediol (1,3-PD). Currently, the polyalcohol is expensively obtained from petrochemical origin, but it is of growing interest mainly for modern fibre synthesis. Within the European Union-funded research project BIODIOL, an integrated bioconversion process for 1,3-PD production from raw glycerol is developed. Untreated glycerols from biodiesel industry are used as the main substrate. A new, wild-type strain and a tailor-made strategy lead to 1,3-PD concentrations up to 100 $g \times L^{-1}$.

The process development is driven by both economical and ecological aspects. The utilisation of cheap substrate sources such as biodiesel and starch industry supports the sustainable approach. Thereby, the production costs of 1,3-PD can be reduced to an efficient and competitive level.

Keywords: 1,3-propanediol, glycerol fermentation, bioconversion, BIODIOL

Zusammenfassung

Entwicklung eines integrierten Biokonversionsprozesses zur Herstellung von 1,3-Propandiol aus Rohglycerin-Wässern

Die Verwertung des in zunehmendem Maße anfallenden Glycerins als Nebenprodukt in fettverarbeitenden Prozessen stellt für die damit verbundene Industrie ein immer größeres Problem dar. Die Aufarbeitung dieses Glycerins zur Pharmaqualität ist energieaufwändig und vor allem für die Biodiesel-Produzenten keine wirtschaftliche Alternative. Eine andere Möglichkeit zur Aufwertung von Rohglycerin ist die mikrobiologische Umwandlung in die wichtige Zwischenchemikalie 1,3-Propandiol (1,3-PD). Dieser Polyalkohol wird derzeit nur kostenintensiv petrochemisch hergestellt, trifft aber angesichts seiner hervorragenden Eigenschaften auf steigendes Interesse vor allem in der Herstellung moderner Kunstfasern. Im Rahmen des von der Europäischen Union geförderten Forschungsprojektes BIODIOL wird ein integriertes Biokonversionsverfahren zur Herstellung von 1,3-Propandiol aus Rohglycerin entwickelt. Die verwendeten Substrate sind vorrangig unaufgearbeitete Glycerine der Biodiesel-Industrie. Ein neuer, gentechnisch unveränderter Stamm und eine maßgeschneiderte Prozessführung erlauben Produktendkonzentrationen von bis zu 100 $g \times L^{-1}$ (1,3-PD).

Die Verfahrensentwicklung erfolgt unter besonderer Berücksichtigung der ökonomischen und ökologischen Aspekte. Die Verwendung preisgünstiger Substrate der Biodiesel- und Stärkeindustrie unterstreicht die Nachhaltigkeit des Prozesses und ermöglicht, die Produktionskosten von 1,3-PD auf ein wirtschaftliches Niveau zu senken.

Schlüsselworte: 1,3-Propandiol, Glycerin-Fermentation, Biokonversion, BIODIOL

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1 Introduction

The bioconversion of glycerol to 1,3-propanediol (1,3-PD) was characterised in 1996 as "remaining messy and unattractive" by Shell Chemical (Chuah 1996). A lot has changed since on both the glycerol and the 1,3-PD site.

The glycerol market is developing and volatile. This was already proposed in 1992 by Deckwer (Deckwer et al, 1992) and is supported by recent figures. Procter & Gamble (Appleby 2003) is expecting a quantity of 840 ktons of glycerol to be produced as a by-product in 2005, further trends clock at almost 1200 ktons in 2010. More than half of this glycerol will be obtained from biodiesel production. Per ton of methyl ester, 100 kg of raw glycerol is obtained as a waste product. Due to the rapidly developing "green fuel" industry both in Europe and the United States, the amount of glycerol as by-product from transesterification constantly increases. However, not to the biodiesel companies' and agriculture's benefit: the massive glycerol production also forces a collapse in its market price. With less income from raw glycerol sales, it gets more difficult to run a profitable biofuel business. To keep profitability stable, an efficient use of the "waste" product glycerol is necessary.

One way to valorise glycerol from biodiesel industry is the conversion into a high-price chemical, such as 1,3-propanediol. 1,3-PD is a polyalcohol with various applications mainly in polyester but also in solvent or antifreeze preparation. It has early been determined as a "viable industrial chemical intermediate" by the leading chemical producer, Shell (Shell 2002). The polyalcohol has proved to be a benefit-increasing part of the promising new polymer PTT (polytrimethylenterephthalate), commercialised by global players such as Shell (Corterra®, mainly for carpet fibres) and DuPont (Sorona®, mainly for functional wear). Recently, the major part of 1,3-PD is produced petrochemically from the harmful acrolein or ethylenedioxide. Both processes are low in yield (not more than 43 %), expensive and not fitting the innovative idea of sustainable polymers. The chemical production price also limits the use of 1,3-PD for PTT, also the re is a high demand: in the fourth quarter of 2004, a 95000 t/a PTT-plant in Quebec, Canada, started production from chemically gained 1,3-PD. Increasing demand is expected once the main chemical for PTT can be produced more efficiently.

One way for highly efficient production is the bioconversion from low-value chemicals like glycerol. However, this approach is not new, it has been focussed and described in detail by various research groups (Abbad-Andaloussi et al, 1995; Barbirato et al, 1998; Biebl et al, 1999). Different microbial strains such as *Clostridium*, *Klebsiella* and *Citrobacter* species produce 1,3-propanediol from glycerol as a general part of their NADH-regeneration metabolism (Biebl et al, 1999). The maximum reported 1,3-propanediol concentration obtained with wild-type strains was reported by Zeng (Zeng et al, 2002) as 86 $g \times L^{-1}$.

Following the pathway determination, well-known organisms like *E. coli* were genetically modified to produce 1,3-PD also from cheap glucose syrup (Nakamura et al, 2003), winning DuPont and Genencor International a Presidential Green Chemistry Challenge Award in 2003 (Ritter 2003), but not yet realised as a large-scale production plant.

Still - keeping in mind the worsening glycerol situation - the low-price substrate raw glycerol in various qualities obtained from biodiesel production was evaluated for the bioconversion to 1,3-propanediol by different teams (Bock 2004; Gonzalez-Pajuelo et al, 2004; Papanikolaou et al, 2004). All groups, employing *Clostridium* species as these were responding best to non-refined substrate sources, did observe an inhibition effect and reduced 1,3-propanediol concentrations compared to refined glycerol conversions. The effects are supposed to be due to heavy metal presence and fatty acid or soap residues (Wittlich 2001).

Any microbial 1,3-propanediol-production must prove its quality in comparison to the existing chemical paths. The major cost factors for such a process are the raw material costs with a share of more than 50 % (Grothe 2000). Wittlich (Wittlich 2001) showed that at a glycerol price lower than 259 €/t, bioconversion is profitable and thus competitive to petrochemical production. Following the development of crude oil prices, this equation is likely to support bioprocessing in the next years massively.

Within the European Union funded project BIODIOL, an integrated bioconversion of raw glycerol to 1,3-propanediol is being developed and evaluated. The sustainable process development is based on non-modified strains and uses waste water effluents from biodiesel and starch industry, meeting the economical, ecological, social and ethical requirements of a modern biotechnological production.

2 Microorganism, media and cultivation parameters

All experiments were carried out with the robust *Clostridium* species IK124, screened at the FAL. IK124 is a strictly anaerobic, wild-type (risk class 1), spore-forming bacterium. The main products from glycerol metabolism are 1,3-propanediol, butyric and acetic acid. The metabolic pathways are expected to be similar to those described by Biebl. IK 124 has a very high tolerance towards oxygen, substrate, products and process interferences, making it ideal for an industrial process.

For reference fermentation, IK124 was cultivated on a half-synthetic mineral salt medium containing: KH_2PO_4 2,71 gL^{-1}, NH_4Cl 2,69 gL^{-1}, $MgSO_47H_20$ 0,12 gL^{-1}, $CaSO_42H_20$ 0,017 gL^{-1}, $FeSO_47H_20$ 0,01 gL^{-1}, yeast

extract (microbiological quality, MERCK KgaA) 5 gL^{-1}, resazurine (0,1 % [V/V]) 0.5 mlL^{-1}, L-cysteineHCl 0,1 gL^{-1}, trace element solution DSMZ 144 1% [V/V], refined glycerol 10-130 gL^{-1}. Variations were made substituting refined glycerol by raw glycerol waters (glycerol concentration adjusted) and yeast extract by potato nitrogen concentrate (PNC, obtained from AGRANA, Austria). Yeast extract was varied for the studies as well. Feed contained 80 % [w/V] glycerol and 40 gL^{-1} yeast extract. Pre-cultures and liquid stock cultures were prepared using 50 ml glass vials and mineral salt media. Lab scale cultivation was carried out under nitrogen atmosphere at pH 7.0 and T 32 °C. Fed-batch fermentation was done in an online-controlled 0,7 L parallel fermenter system with a cell-free sampling probe (FISP®, Flownamics), connected to HPLC (BioRAD Aminex 87H column, mobile phase 5mM H_2SO_4, 60 °C, 0.7 mL/min flow) for acquisition and feedback regulation. All data acquisition and regulation was made using the software DasyLAB 5.0 (datalog, Moenchengladbach). For repeated-batch, a simpler 0.4 L fermenter without online sampling was used. Both 0,4 L and 0,7 L fermentation was pH-controlled by addition of 5 or 10 M NaOH. Manual samples were withdrawn at process relevant stages.

3 Fermentation strategy

The optimisation of fermentation is mainly driven by the need to overcome inhibitions of both substrate and products. The impact of 1,3-propanediol and the organic acids produced during fermentation was estimated earlier (Colin et al, 2000; Colin et al, 2001). We could also find that a "cocktail" of all accumulating media compounds and the remaining substrate limits the final product concentration of 1,3-PD: The process tends to stagnate not only due to reaching the limiting concentrations of all of the components, but at a certain presence of various inhibitors.

Several process strategies were tested to overcome inhibition and to increase the final 1,3-PD concentration. Zeng (Zeng et al, 2002) outlined fed-batch as ideal, which combines high product concentrations from batch experiments with a slight glycerol excess beneficial for quick production from continuous cultivation. Feeding is generally organised by using the proportionality between growth, acid and 1,3-propanediol formation: glycerol consumption can be met by synchronising the feed pump and the addition of base. We have further refined this system, combining a low base-driven glycerol addition with constant online measurement of glycerol and a feedback regulation. The construction of the glycerol set value and its control is also connected to the recent 1,3-PD amount and lowered at late process stages (more then 60 $g \times L^{-1}$ 1,3-PD). Using this strategy and also the high tolerance of our strain IK124 towards substrate and its metabolites, we could reach 87 $g \times L^{-1}$ final 1,3-PD concentration at a productivity of 2,2 $g \times L^{-1} \times h^{-1}$ (reference fermentation at 25 $g \times L^{-1}$ starting glycerol concentration). This is the highest 1,3-PD concentration reported so far using a wild-type strain. By doubling the yeast extract both in media and feed, even product concentrations up to 100 $g \times L^{-1}$ were obtained. In both variants, yields ate at 65 % [mol/mol].

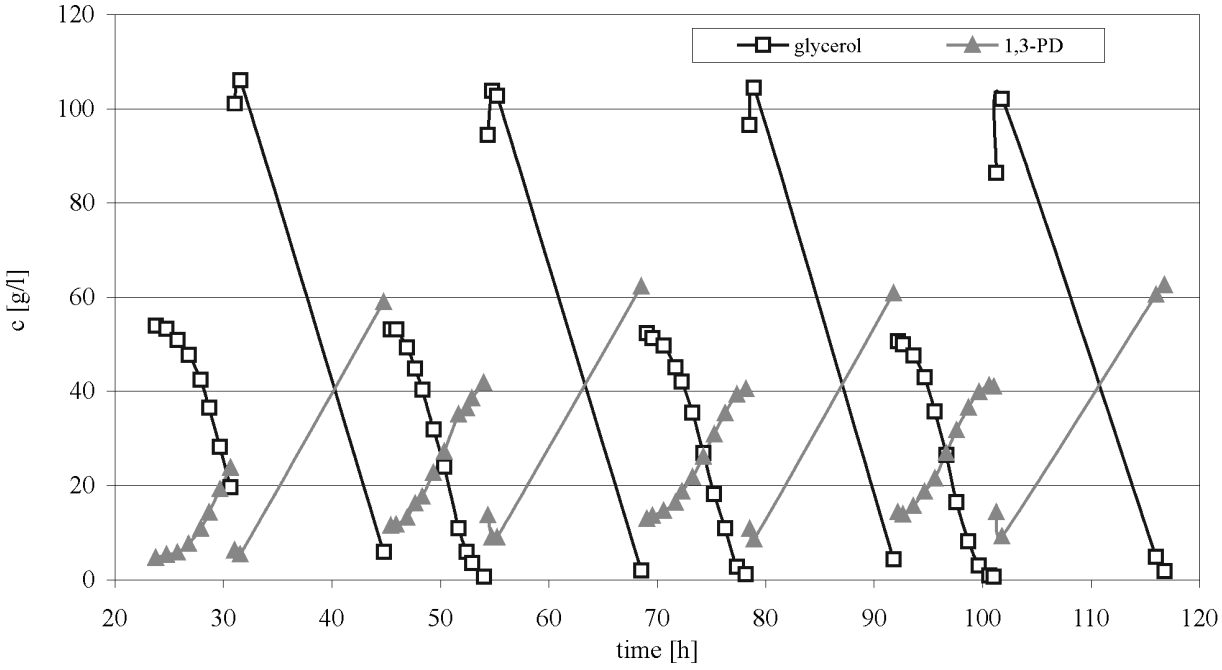

Fig.1:
1,3-propanediol production and glycerol consumption in representative repeated batches (cultivation on beech wood chippings)

The influence of pre-culture quality on the recent fermentation remains a possible source of error, thus the 700 ml equipment was set-up as a parallel two-fermenter system. Variations can herewith be judged in comparison to a reference. To further avoid pre-culture effects, repeated-batch mode was developed for high throughput tests. This also is a process strategy to meet industrial and economical needs. Repeated-batch was carried out using either suspended or adsorbed cells. Both free cells with a hold back of 10 % fermentation broth and cells adsorbed on beech wood chippings cause a constant growth without a lag phase after medium exchange and therefore a productivity increase up to 3,6 $g \times L^{-1} \times h^{-1}$. Fig. 1 shows the substrate and product graphs of eight significant batches taken from a repeated-batch on beech wood chippings. The substrate concentrations do, due to better handling, vary between day (55 g/l glycerol) and night (110 g/l glycerol) batch. The repeated-batches can be run up to a month (at 2-3 batches a day) without any product reduction or changes in cell behaviour and product spectrum. As its biggest benefit, this strategy resulted in excellent culture stability towards oxygen, contamination, high substrate concentrations or process interferences. Cells in non-limiting conditions tolerate refined glycerol without growth inhibition up to 130 g/l. Fermentation can be carried out with only little effort on anaerobic conditions. In combination with less pre-culture preparation effort and no additional costs, repeated-batch is a desirable process mode for industrial production.

4 Utilisation of cheap substrate sources

One fundament of a sustainable process is the use of substrates from renewable origin instead of fossil resources. Therefore, the use of non-refined glycerol phase produced as a by-product from biodiesel as well as and starch or sugar industry effluents as the complex media component were focussed.

Glycerol waters of several origins (fatty acid splitting, saponification and transesterification) were tested for the biotechnological 1,3-propanediol production (Bock, 2004). The greatest ecological and also microbial benefit is obtained from the use of biodiesel glycerols. Depending on their quality, effects like less productivity, reduced final 1,3-propanediol concentration and a prolonged lag-phase can be observed. This is mainly due to the presence of an organic phase (OP), containing soaps, salts and other organic residues. Contrary to earlier expectations, high methanol contents (exceeding 5 % [w/w] in direct waste effluents from biodiesel) are not a major inhibition factor and do not need to be evaporated prior to fermentation.

Non-purified glycerol waters differ by origin, campaign or production procedure, and might require either a tolerant microorganism or a pre-treatment step. A very effective way is the industrial removal of OP: glycerol waters are treated with organic acids for phase separation, the resulting glycerol phase can be further purified. This brings a tremendous performance benefit; especially the lag-phase is shortened to approximately 10 h (untreated: 20-70 h, reference: 4-6 h). Fig. 2 shows the comparison of refined (reference), untreated and two organic-acid treated glycerol waters used for online-controlled fed-batch fermentations. After treatment, no significant differences between glycerol types from various biodiesel plants are visible. An alternative treatment method using cheap technical HCL was developed. This method also has the lag-phase-shortening effect as observed by industrially treated glycerols (data not shown).

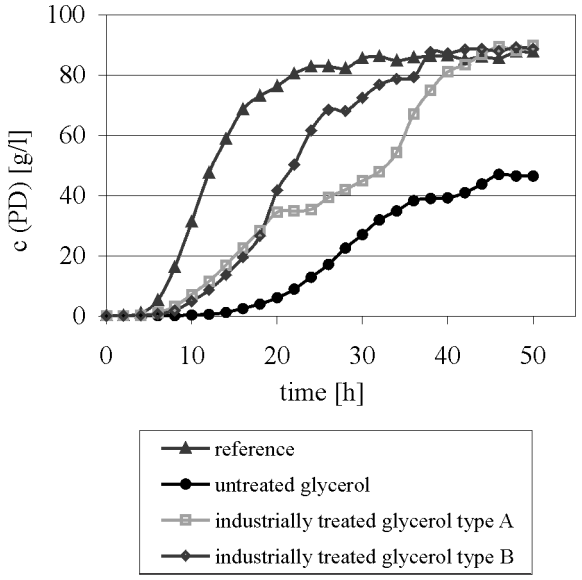

Fig. 2:
cultivation on refined, untreated and two industrially treated glycerols

Table 1:
influence of the media composition on the bioprocess factors product concentration, yield and productivity

experiment	1,3-PD-concentration [g/L]	yield (% [w/w])	overall productivity [g/(L×h)]
reference	87,7	54	1,9
raw glycerol usage	80,1	56	1,8
PNC usage	80,0	56	1,4
combination raw glycerol/PNC	77,5	56	1,2

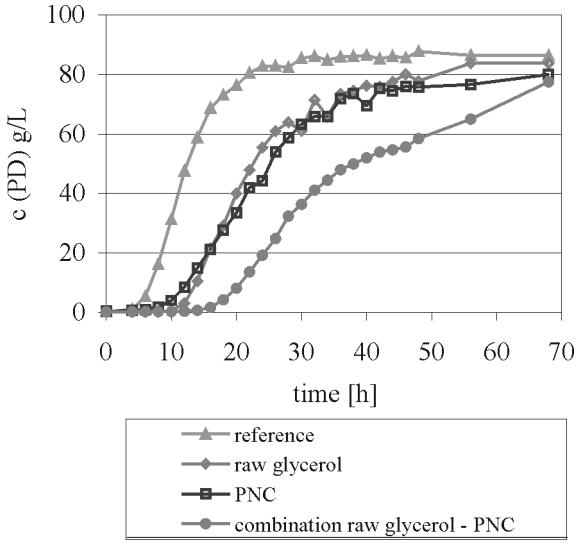

Fig. 3:
influence of the media composition on 1,3-PD formation

As a second step, the substitution of yeast extract by wastewater effluents was tested. Different well-known fermentation additives such as corn steep liquor or mother liquor have been evaluated, neither of which proved to be sufficient for the bioconversion. The most promising results were obtained using potato nitrogen concentrate (PNC), a cheap effluent from starch industry. Due to low amino acid and high nitrate content, PNC has both limiting and inhibiting effects. An optimum addition amount of PNC has been found at approximately 50 g/l (of which 20 g/l in media, 30 g/l in feed), giving 92 % of final product concentration (80 g/l 1,3-PD) and slightly increased yield (67 % [mol/mol]).

The combination of raw glycerol and PNC has been tested in fed-batch fermentation at the above-mentioned ideal PNC amount and 30 g/l starting raw glycerol concentration. The influences of both substrates add up to a prolonged lag phase of about 16 hours and product loss of 10 % (77,5 g/l 1,3-PD).

Table 1 and Fig. 3 summarise the bioprocess performance using both cheap substrate sources.

5 Economical aspects

To interpret the results from bioconversion, an economical evaluation of the integrated process was made for a medium size technical 5 ktons/year production plant. It could be outlined that the price of microbiological 1,3-PD production is mainly influenced by process parameters such as yield, final 1,3-PD concentration and fermentation time (see Fig. 4). These parameters have an impact on all cost aspects; for example a prolonged fermentation time will increase the number of fermentation vessels required for constant production. For further details, the production costs were split into six categories and are shown for a reference process in Fig. 5.

More then 37 % of the total production costs (capital, personal, energy and waste) are barely flexible, but will directly depend on the bioprocess performance and the influence factors outlined. 13 % of the costs are pre-culture costs, which can be reduced by a tailor-made strategy. The repeated batch mode (part of each batch is hold back,

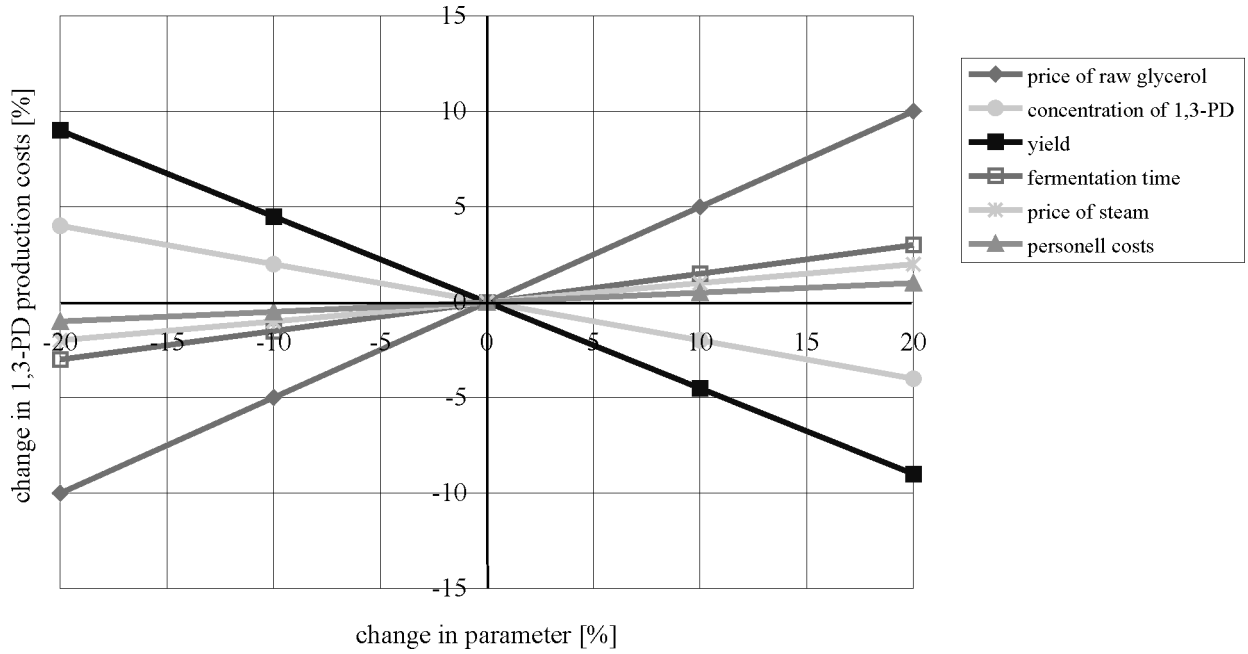

Fig. 4:
sensitivity analysis of 1,3-PD production parameters

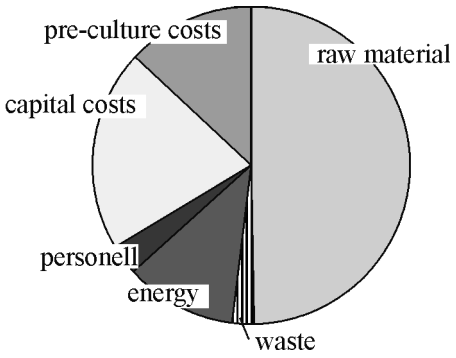

Fig.5:
parameters' share of the total 1,3-PD production cost

new inoculation only after 10 batches) allows the reduction of inoculum preparation costs to only 3 % total share (see also Fig. 6).

The largest cost share of a reference process are the raw material cost with 50 %. Their major parts are refined glycerol (app. 600 €/ton glycerol) and yeast extract (minimum 3500 €/ton for technical quality). The BIODIOL process is to be set up on using only low-quality substrates such as the evaluated raw glycerol phases and PNC. The price for industrially treated raw glycerol is constantly lowered, and it should be far below the 259 €/ton characterised by Wittlich (Wittlich 2001) as economically reasonable.

Combining all reduction possibilities and the recently obtained 1,3-PD production performance of IK 124 on non-refined substrates, cost can be reduced by more than 20 %. Assuming that it is possible to run a bioconversion with cheap substrates at the performance of a reference process, 33 % 1,3-PD production expenses can be saved (Fig. 6).

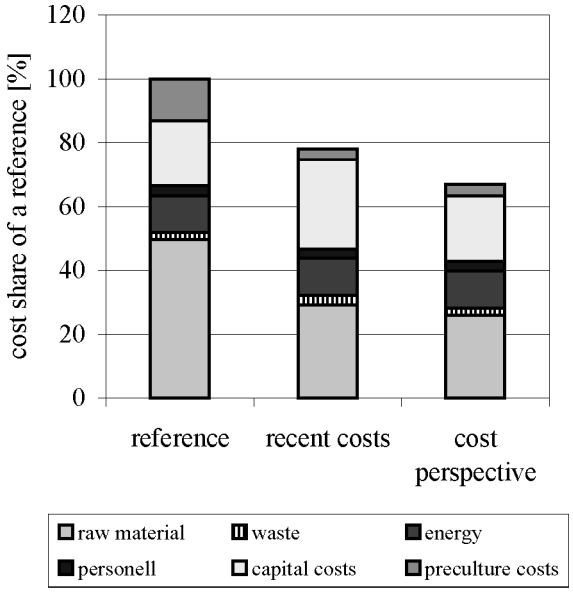

Fig. 6:
reference costs, recent reduction and potential (in percent of a reference)

6 Discussion

Microbial production of 1,3-propanediol from renewable resources can be competitive to chemical production routes, if both its complex cost structure is well evaluated and an optimisation has produced an ideally running integrated bioprocess. It must not solely be based on cost evaluation, but also on sustainability aspects.

As shown, major improvements could be made towards this aim. The BIODIOL process can be run without chemicals based on crude oil and herewith ecologically reasonable; it is as many bioprocesses characterised by high yield and high selectivity towards the desired product; it does not interfere with ethical aspects or presents a safety risk as IK 124 is a wild-type strain and - as a major aspect - is presents a perspective for the biodiesel industry to face the growing glycerol "waste" problem.

The evaluation of different BIODIOL process routes was made balancing the low costs of non-purified substrates and their effects. It has been focussed to find an optimised strategy where as much product as possible is obtained in the shortest time at lowest prices. Using an industrially treated glycerol and PNC as a yeast extract substitute, this focus has been met. Work on both microbial and technical aspects will continue to make a process on cheap material as productive as a reference. Further work will include the development of a simple strategy that is easy to understand, accept and implement by the biodiesel industry.

The integrated bioprocess development and economical evaluation of BIODIOL also includes partner's work on substrate supply and -purification, glycerol sensors, product recovery and immobilisation, which is not presented here. The BIODIOL project (QLK5-CT-2002-01343) is funded by the European Commission within the 5th Framework Program. The EU is not responsible for the content of this publication.

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