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Cleaner production of phenylacetylcarbinol by yeast through productivity improvements and waste minimisation

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Abstract: Phenylacetylcarbinol (PAC) in its laevo-rotatory chiral form (L-PAC) is a precursor for the synthesis of L-ephedrine and D-pseudoephedrine, two pharmaceuticals with nasal decongestant properties. L-PAC is generated biologically through the pyruvate decarboxylase (PDC)-mediated condensation of added benzaldehyde with acetaldehyde generated metabolically from feedstock sugars via pyruvate. Some of the added benzaldehyde is converted through the action of alcohol dehydrogenase(s) to benzyl alcohol, an undesired by-product. L-PAC extracted from the fermentation broth is converted chemically by hydroamination in the presence of methylamine and hydrogen to L-ephedrine, and then by isomerisation to D-pseudoephedrine.

We have employed a dual approach strategy to enhance the ratio of product to by-product generated and to minimise the waste treatment burden of the spent fermentation broth. Benzaldehyde delivery to the fermentation has been modified to ensure that sufficient raw material is available, together with pyruvate, during peak periods of PDC activity, and that benzaldehyde is less available during periods of high alcohol dehydrogenase activity. The inorganic content of the spent fermentation broth has been reduced substantially by the partial substitution of raw sugar for molasses in the medium, with a reduction of molasses content by 60% resulting in an increase of PAC yield of 20% and increased specific productivity. Whilst using the raw sugar-molasses medium we have re-evaluated the contribution of other impure fermentation feedstocks such as dried whey, and corn steep liquor, and found that both can be eliminated without losses in PAC production. Further work on the optimisation of the concentrations of carbohydrate, nitrogen and phosphate in the fermentation has been conducted and has led to further productivity increases, together with reduced waste generation, resulting in an L-PAC process which is considerably "cleaner" than the parent process.

INTRODUCTION

Pyruvate decarboxylase (PDC) catalyses the conversion of pyruvate to acetaldehyde with the resultant loss of a molecule of CO_2. The reaction requires the co-factors thiamine pyrophosphate (TPP) and magnesium ion (Equation 1).

$$\underset{\text{Pyruvate}}{CH_3COCOOH} \xrightarrow[TPP,mg^{2+}]{\text{Pyruvate decarboxylase}} \underset{\text{Acetaldehyde}}{CH_3CHO} + CO_2 \qquad (1)$$

PDC then catalyses the condensation of acetaldehyde and pyruvate, forming acetoin (Equation 2):

$$\underset{\text{Pyruvate}}{CH_3COCOOH} + \underset{\text{Acetaldehyde}}{CH_3CHO} \xrightarrow[TPP,\ Mg^{2+}]{\text{Pyruvate decarboxylase}} \underset{\text{Acetoin}}{CH_3CHOHCOCH_3} + CO_2 \qquad (2)$$

After the formation of acetaldehyde according to Equation 1, PAC is formed, by analogy with Equation 2, by the condensation of added benzaldehyde and acetaldehyde (Equation 3):

$$\underset{\text{Pyruvate}}{CH_3COCOOH} + \underset{\text{Benzaldehyde}}{C_6H_5CHO} \xrightarrow[TPP,Mg^{2+}]{\text{Pyruvate decarboxylase}} \underset{\text{PAC}}{C_6H_5CHOHCOCH_3} + CO_2 \quad (3)$$

The pyruvate consumed in the reaction is commonly generated by the yeast via glycolysis and is allowed to accumulate exogenously during the exponential phase of growth. Where the yeast is used as a catalyst only, and is not freshly grown, added pyruvate can also be utilised. Commercial processes are generally divided into two stages: a first stage where the yeast is grown followed by a bioconversion stage where benzaldehyde is added and PAC produced.

Acetaldehyde is also a substrate for alcohol dehydrogenase (ADH). Like PDC, ADH isoenzymes have also been shown to display reactivity towards benzaldehyde, reducing it to benzyl alcohol according to the following reaction (Equation 4):

$$\underset{\text{Benzaldehyde}}{C_6H_5CHO} + NADH + H^+ \xrightarrow{\text{Alcohol dehydrogenase}} \underset{\text{Benzyl alcohol}}{C_6H_5CH_2OH} + NAD^+ \quad (4)$$

The production of benzyl alcohol is undesirable in the commercial production of PAC.

Nutrient effects in PAC production and medium formulation

It is not uncommon for industrial fermentation media to contain one or more complex materials as sources of carbon and nitrogen. They are generally inexpensive and readily available and can also act as sources of vitamins, ionic nutrients and growth factors (ref.1). There are potential disadvantages associated with the use of complex materials in fermentation media including: the presence of compounds in potentially inhibitory concentrations (refs.2,3,4), the presence of unmetabolisable compounds (refs.5,6) and the presence of compounds which chelate or sequester ionic nutrients (refs.3,7). Another potential disadvantage is the induction of phenomena such as diauxic growth which can occur when more than one form of a nutrient is available. Diauxic growth was attributed to the scale-up problems experienced with the PAC process(ref.8) and was overcome by modifying the fermentation medium and altering operational variables. Optimisation of individual nutrients in industrial medium comprised of complex materials is difficult because the alteration of the concentration of the complex material changes the concentrations of all its individual components.

Alternatively, defined media may be used, with the advantages of constant composition and the exclusion of toxic or inhibitory compounds. However, the use of a defined medium on an industrial scale may limit the commercial viability of the process (refs.1,9). On a small scale, PAC yields of up to 22 gl^{-1} have been achieved using a defined medium compared with 13 gl^{-1} in undefined medium (ref.10). Others (ref.11) achieved higher PAC yields (4.7 - 5.6 gl^{-1}) when additional yeast hydrolysate and/or CSL was included in the medium compared with a basal medium containing a low yeast hydrolysate concentration (2.5 gl^{-1} PAC). However, the basal medium (ref.11) was very simple, containing no vitamins or ionic nutrients.

The work conducted to date on the effects of nutrition in PAC production has been limited (see ref.10,11,12). Noronha and Moreira (ref.13) have developed a PAC production medium (details not published) with a glucose concentration of approximately 100 gl^{-1} (ref.14) to stimulate the fermentative activity of *S. cerevisiae*. Others, all employing *S. cerevisiae*, have used media ranging from fully defined media (refs.15,16) to very simple media containing nothing more than molasses and urea and/or phosphate (refs.17,18).

Substrate choice is essentially dependent on the process used with glucose or sucrose (as pure sucrose or molasses) commonly used when biomass is grown *in situ* (ref.11,18,19) or when an acclimatisation period was used for purchased biomass (refs.19.20.21). When the biomass is used essentially as a catalyst, pyruvate is generally used and has been found to produce higher and more reproducible PAC yields because the regeneration of NADH, and hence the production of benzyl alcohol, is limited (ref.19).

Molasses has been successfully employed in media of varying composition and degrees of complexity for the production of PAC. Possibly one of the simplest media comprising 10-15% molasses and 0.2 - 0.25% urea (ref.18) achieved PAC yields of up to 5 gl^{-1} with a *S. cerevisiae* strain. Another very simple medium contained

6% molasses, 3.5% crude sugar and 0.05% $MgSO_4.7H_2O$ (ref.22) while one of the more complex medium formulations contained 20% w/v molasses and was supplemented with ammonium, phosphate, yeast extract and magnesium (ref.23).

Complex nitrogen sources including corn steep liquor (CSL) and yeast hydrolysate (ref.11), yeast extract (refs.19,23), peptone (refs.19,20,24) have been used in fermentation media for the production of PAC. Whey is also a component of the medium used for this work, and is commonly used in fermentations as a source of carbon in the form of lactose (refs.1,4,9). There are, however, a limited number of yeasts capable of utilising lactose not including *Candida utilis*, the organism used in this study. Whey has also been used as a source of nitrogen (ref.1), phosphorus, vitamins and minerals (ref.6), although supplementation with each of these from other sources may still be required.

Substrate dosing

One of the simplest and least costly methods for reducing the biomass exposure to inhibitory concentrations of benzaldehyde is to regulate the dosing regime to limit the benzaldehyde concentration in the medium at any time. During the development of a semi-continuous process for PAC production using *S. cerevisiae*, it was found (ref.25) that the continuous addition of benzaldehyde over 6 hours enabled up to double the amount to be added compared with intermittent dosing, and that the PAC yield was increased by up to 2.5 times. A greater proportion of benzaldehyde was converted to PAC when the benzaldehyde was fed continuously rather than intermittently. The use of a continuous feeding protocol appeared to reduce the sudden inhibitory effect of benzaldehyde and led to more efficient conversion of benzaldehyde to PAC. It has often been reported that complete conversion of benzaldehyde to PAC is not possible in whole cell systems because of the formation of by-products, namely benzyl alcohol and benzoic acid (refs.15,16,21,25,26,27,28,29). Benzyl alcohol is the main by-product of the PAC process with reports of up to 50% of added benzaldehyde being converted to benzyl alcohol (ref.27). For every gram of benzyl alcohol produced, the PAC yield is potentially reduced by 1.4 g. It is clear that minimising the formation of benzyl alcohol production during PAC fermentations could result in significant increases in PAC yield.

There has been some success in reducing the amount of benzaldehyde converted to benzyl alcohol by modifying benzaldehyde dosing protocols to variously enhance PDC activity and reduce ADH activity. As for many other enzymes, ADH and PDC activities are related to substrate concentrations. Formation of benzyl alcohol by acetone powders of brewer's yeast occurred (ref.26) when the benzaldehyde concentration was in excess of the pyruvate concentration. Alternatively, by maintaining benzaldehyde concentrations greater than 4 mM, *S. cerevisiae* PDC activity was higher than ADH activity (ref.15). Baker's yeast ADH was much more sensitive than PDC to inactivation by benzaldehyde, with inactivation at concentrations as low as 0.2 gl^{-1} (ref.21). The potential use of high benzaldehyde concentrations to lower benzyl alcohol yields was therefore recognised but it was noted (refs.20,21,24) that the inhibition or inactivation of ADH by benzaldehyde could also restrict the regeneration of NAD^+ which was required for the production of pyruvate from hexoses.

Waste Minimisation

The waste treatment requirements of spent fermentation broth have a potentially large impact on the cost-effectiveness of the process at an industrial scale due to the cost of either discharging the waste to sewer, or the cost of treating the waste on-site prior to discharge. In the Melbourne metropolitan area, industrial waste is discharged to the sewerage system and is subject to a trade waste agreement. Disposal costs are based on a variety of factors including volume, temperature, organic strength, nitrogen and sulphate content and is based on cost recovery (ref.31). Treatment of industrial waste prior to discharge is common and involves the manufacturer installing waste treatment facilities, sometimes at considerable expense.

The organic content is readily treated although the cost increases as the strength of the waste increases and some materials, such as colour compounds, are more difficult to remove. Nutrients, i.e., nitrogen and phosphate, are harder to remove than organic load and are often limiting factors in the eutrophication of waterways. Nitrogen is removed mostly in a gaseous form by biological methods but it exerts a large oxygen demand and increases treatment costs. Phosphorus, once it enters a system, is difficult to remove because it

does not exist in gaseous forms. Chemical removal techniques are available, e.g., flocculation and sedimentation with ferric chloride, but lead to the generation of iron- and phosphate-rich sludge which is generally disposed of in land fill. An alternative approach to reducing the organic and nutrient content of the waste is by reducing the amounts which are added in the production process.

The aim of this study was to investigate the effect of simplifying the medium content on the production of PAC and benzyl alcohol, and the resulting effect of modifications on the COD of the spent medium. These aims were achieved by the progressive removal or reduction of medium components and comparing the yields, specific productivity, and COD of the spent broth.

METHODS

Preparation of inoculum and fermentation medium

The culture of the *C. utilis* strain used for this project was supplied by ICI Australia Operations Pty. Ltd. (ICI) and maintained by bimonthly subculture onto fresh Malt Extract Agar (MEA) slopes.

The medium used for both the growth of inoculum and for the fermentation medium was based on the medium used by ICI and contained a variety of complex materials including B Boil-Out molasses (BBO), a by-product of the sugar-cane refining process, and corn steep liquor (CSL) as well as urea and KH_2PO_4. All medium was prepared from technical grade components.

Fresh fermentation inoculum was used for all fermentations, and was prepared by inoculating 100 ml of sterile medium with the contents of a fresh MEA slope. The flask was incubated for 24 hours on a shaking incubator.

Ten litres of medium was used for the growth phase of fermentations conducted in a Biostat E fermenter (B.Braun, Germany), and 1.5 l of medium was used for fermentations conducted in Biostat B fermenters (B.Braun, Germany). In addition to the components mentioned above, the fermentation growth medium also contained glucose syrup. Forty ml of inoculum was used to inoculate the 10 l fermentations, 10 ml was used to inoculate the 1.5 l fermentations.

All fermentations were run non-aseptically in two stages, a growth phase and a bioconversion phase, using the standard ICI medium and batch protocol.

When the residual glucose in the medium was less than 10 gl^{-1}, as determined by visual inspection of glucose Tes-tape® (Eli-Lilly) or Clinistix® (Ames), the bioconversion phase of the fermentation was initiated by the addition of extra BBO, urea and KH_2PO_4, as well as whey, $MgSO_4$ and thiamine. The temperature was reduced, the pH was raised and the aeration rate was reduced by half. An aliquot of benzaldehyde was added to the fermenter.

Two hours after the initiation of the bioconversion phase extra BBO was added to the fermenter, and the continuous addition of benzaldehyde (total of 15 $ml l^{-1}$) was commenced. A peristaltic pump was used to add benzaldehyde to both the Biostat E and Biostat B fermenters. The point at which the extra carbohydrate was added and the benzaldehyde feed was started was referred to as Pump Start (PS). Forty hours after inoculation of the fermenters, final samples were taken and the fermentations were terminated.

Sampling of broth and analysis of samples

Cell dry weight was determined immediately after sampling by measuring the absorbance of filtered, washed and diluted samples at 600 nm.

Samples used for the determination of pyruvate and reducing sugar concentrations were centrifuged for 10 minutes at approximately 1200 *g*, and the supernatant stored at -20°C prior to analysis.

Reducing sugar concentration was determined using the dinitrosalicylic acid (DNS) method. A new set of

standard curves was prepared for each set of samples. Absorbance of standards and samples was measured at 540 nm and 580 nm.

A method for single reagents (Pyruvic acid - UV method, Boehringer Mannheim) was used to determine pyruvate concentrations in fermentation samples. Fermentation samples were assayed in duplicate and diluted if necessary.

Samples used for determination of benzaldehyde, benzyl alcohol, PAC and Chemical Oxygen Demand (COD) were immediately centrifuged for 20 minutes at 13 680 *g* in a centrifuge cooled to 4°C (Sorvall RC-5C, Du Pont, U.S.A.) and decanted into clean containers prior to further treatment. Samples used for determination of COD were stored at -20°C until analysed.

Samples used for the analysis of benzaldehyde, benzyl alcohol and PAC were extracted with an equal volume of dichloromethane containing dodecane as an internal standard. The extract was held at -20°C until analysis within a week of preparation.

Gas liquid chromatography (GC) was used to determine the concentrations of benzaldehyde, benzyl alcohol and phenylacetylcarbinol (PAC) in the fermentation broth. Samples were analysed using a Shimadzu GC 8A gas liquid chromatograph (Kyoto, Japan) fitted with a 12 m polyimide-coated capillary column with BP1 stationary phase, 0.22 mm ID, 0.1 micron film (SGE, Melbourne, Australia) and coupled to a recorder-integrator (Shimadzu Chromatopac C-R3A). Injector and detector temperatures were maintained at 250°C, and the oven temperature was raised from 80°C to 120°C at a rate of 2°C/min immediately after injection of the sample/standard. Standard solutions and samples (0.1 µl) were run in triplicate.

The Chemical Oxidation Demand was determined using a 'Micro COD' procedure approved by the U.S. Environmental Protection Agency in 1980 (ref.32). Samples were extracted with an equal volume of dichloromethane prior to analysis, diluted if necessary, and tested in duplicate. A direct readout of the COD was given.

Microsoft Excel (Version 5.0) Analysis ToolPak was used to undertake regression analysis of the majority of the data. For the investigation of nitrogen and phosphate content, an irregular, fractional factorial experimental design (IF0412), described in Haaland (ref.33), was used. From the results, Normal Plots were constructed, and regression analysis applied to the 'outliers'.

RESULTS

An empirical approach was followed for the investigation of the nutrient sources in the medium, with the exception of the nitrogen and phosphate contents, where a statistical experimental design was used to simultaneously investigate the effect of the nitrogen and phosphate content. Initially, the BBO content was reduced progressively because it formed the major component of the medium, and because regression analysis of the sucrose and ash content (as provided by CSR, suppliers of BBO) indicated that there was no correlation between the two, i.e., the composition of the BBO was highly variable. This variability had a potentially large impact on the outcome of the fermentations.

Effect of BBO on the production of PAC

The approach employed was to progressively reduce the BBO content of the fermentation medium to determine the minimum amount of BBO required to maintain PAC yields equivalent to those from the standard medium. The reducing sugar content of the medium was maintained by fortification with a high purity substrate (raw sugar) because a reduction in added carbohydrate may lead to potentially reduced PAC yield, as a consequence of a lower potential yield of pyruvate. The ready availability of raw sugar, coupled with ease of handling and low cost, made it a logical choice for use as a high purity substrate. Maintenance of the carbohydrate content of the fermentation whilst manipulating the total BBO content independently of other compounds enabled the collective effects of non-carbohydrate compounds (present in BBO) on fermentation

TABLE 1 Length of Time required to exhaust Carbohydrate Supply for Media with varying Ratios of BBO and Raw Sugar

BBO content (%)	Length of growth phase (h)
100	16.15
75	16.0
50	16.5
40	17.0
25	17.25
0	18.0

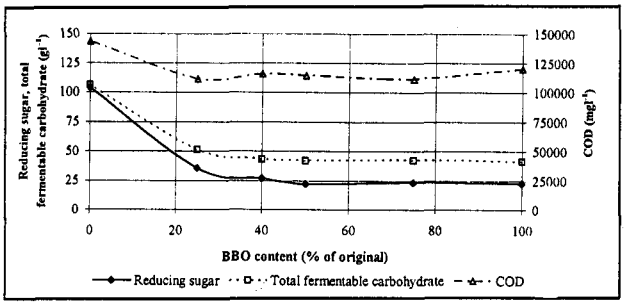

Fig.1 Comparison of COD, reducing sugar and total fermentable carbohydrate concentrations in spent medium with variation in the ratio of BBO and raw sugar in the medium.

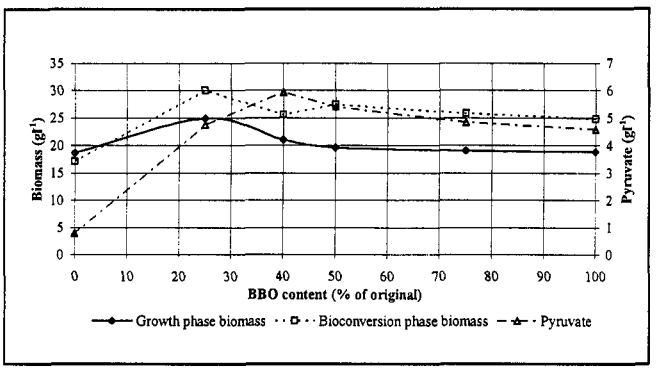

Fig.2 The effect of varying BBO content on biomass and pyruvate concentrations at the end of the growth phase (GP) and on the biomass concentration in bioconversion phase (BP)

TABLE 2 Effect of varyinb BBO Content on the Benzaldehyde Concentration in Spent Medium

% BBO	Residual benzaldehyde (gl^{-1})
100	0.07
75	0.04
50	0.005
40	0.02
25	1.46
0	3.96

performance to be determined. The effect of individual non-carbohydrate compounds was not addressed in the course of the studies reported here.

As previously mentioned, the growth phase was terminated when the carbohydrate supply was exhausted (10 gl^{-1} or less), with the exception of the fermentation containing no BBO which was terminated after 18 h, with a reducing sugar concentration of 40 gl^{-1}, in order to maintain a similar time line to other fermentations. The length of the growth phase increased as the proportion of BBO in the medium decreased (Table 1). On completion of the fermentation, reducing sugar concentrations were similar for all fermentations containing 40% or more BBO, increasing for fermentations containing less BBO (Fig.1).

The maximum biomass and pyruvate concentrations were recorded when the BBO content was equivalent to 25% and 40% of the original amounts respectively, i.e., when the other 75% or 60% of the original carbohydrate content was added as raw sugar (Fig.2).

The benzyl alcohol concentration, measured on completion of the fermentation, was relatively unaffected by the BBO content of the medium (Fig.3). In contrast, the benzaldehyde (Table 2) and PAC (Fig.3) concentrations increased and decreased respectively when the BBO content was 25% of the original or less. The PAC concentration was at a maximum (13.6 gl^{-1}) when the medium contained 40% of the original BBO. The PAC to benzyl alcohol ratio, an indication of the efficiency of bioconversion, followed a similar trend to the PAC yield due to the similarity of benzyl alcohol concentrations between fermentations. The ratio was highest (4.4) when the medium contained 40% BBO (Fig.3).

All fermentations containing 40% or more BBO had similar specific productivities for PAC (q_{PAC}), dropping by a third for medium containing 25% BBO and 60% for medium lacking BBO (Table 3). Comparison of q_{PAC} values for all of the fermentations indicates that the BBO content of the medium can be

reduced to 40% of the original concentration while maintaining a level of specific productivity similar to the fermentations containing a greater proportion of BBO.

The COD of extracted, spent medium was similar for fermentations containing between 25 and 75% of the original BBO content and was strongly correlated with reducing sugar content (r^2=0.87), while the CODs of the media containing 0 and 100% BBO were slightly higher (Fig.2). The higher COD of the spent medium containing raw sugar only was probably due to the increased reducing sugar content of the spent medium. In addition to a reduction in the COD of the spent broth as the BBO content of the medium was reduced, the clarity of the spent broth was also improved, and its colour reduced, although the intensity of the colour was not measured.

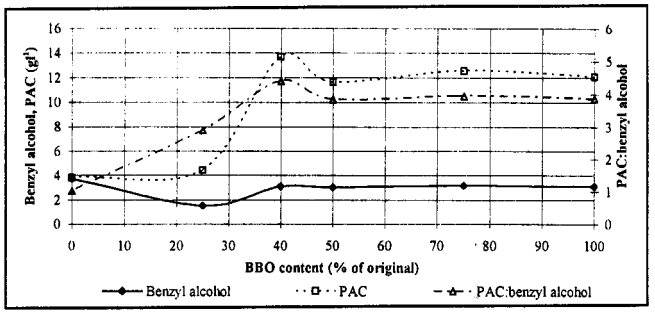

Fig.3 Effect of the BBO to raw sugar ratio of the medium on the production of benzyl alcohol and PAC, and on the PAC to benzyl alcohol ratio

TABLE 3 Effect of varying the Proportions of BBO and Raw Sugar in the Medium on the Specific Productivity of PAC by *C. utilis*.

BBO content	q_{PAC} ($gg^{-1}h^{-1}$)
100	0.012
75	0.012
50	0.0115
40	0.013
25	0.008
0	0.005

The reduction in the length of the growth phase with increasing BBO concentration attests to increasing metabolic activity and is verified by the increases in biomass and pyruvate concentrations when the BBO was 25 - 40% of its original concentration respectively. The decline in both exogenous pyruvate and biomass concentration for BBO concentrations of 50% or more, coupled with the shorter length of growth phase, indicates that pyruvate was converted to volatile or excreted products for which no analysis was carried out, e.g., ethanol. In addition, the long growth phase and large residual reducing sugar concentration suggest that there was a severe deficiency of non-carbohydrate compounds, probably ionic nutrients, in the fermentation lacking BBO. The strong correlation between COD and reducing sugar concentration suggested that reducing sugar concentration was the major contributor to the COD of the spent broth.

There is no obvious explanation, apart from experimental variation, for the reduced benzyl alcohol concentration which occurred when the medium contained 25% BBO. The reduction in benzyl alcohol concentration was not compensated for by increased PAC concentration, and the presence of suboptimal conditions was excluded because the benzyl alcohol yield obtained for the medium containing no BBO was similar to the yields for fermentations containing 40% or more BBO.

The concentration of exogenous pyruvate present at the end of growth phase was considered an important determinant of the final PAC yield obtained given its role as a substrate, and it was correlated moderately (r=0.7) with PAC yield. The highest PAC yield, obtained using 40% BBO and 60% raw sugar in the medium, was an apparent combination of increased pyruvate concentration and a medium formulation favourable to slightly improved PDC activity, given the lower biomass concentration compared with medium containing 25% BBO.

Given that the best PAC yields were achieved for medium containing 40% BBO and 60% raw sugar, that carbohydrate combination was used for the following fermentations. After the reduction of the BBO content, fermentations were conducted to determine whether the addition of CSL, and then whey, was beneficial to the production of PAC.

Effect of the omission of CSL from fermentation growth medium

The presence of CSL was stimulatory to glycolysis with the length of the growth phase being 1 - 1.5 hours shorter when CSL was included in the growth phase medium. However, pyruvate concentrations prior to the initiation of the bioconversion phase were consistently higher when CSL was omitted from the medium (Table 4). Further increases of approximately 2 gl^{-1} were recorded during the 4 - 5 hours before net pyruvate consumption exceeded pyruvate production. The pyruvate concentration was generally below the limit of detection for the assay employed (0.04 gl^{-1}) at the end of the fermentations.

TABLE 4 Comparison of Results for 40% BBO and 60% Raw Sugar Medium containing and omitting CSL

	40% BBO: 60% raw sugar	40% BBO:60% Raw Sugar-CSL
Pyruvate prior to bioconversion (gl^{-1})	5.9	6.7
Final reducing sugar (gl^{-1})	27.0	45.6
Biomass (gl^{-1})	25.6	24.8
Benzaldehyde (gl^{-1})	0.018	0.080
Benzyl alcohol (gl^{-1})	3.1	2.6
PAC (gl^{-1})	13.7	12.8
PAC:benzyl alcohol (gl^{-1})	4.4	4.9
COD (mg^{-1})	115,550	105,117
q_{PAC} ($gg^{-1}h^{-1}$)	0.014	0.013

The inclusion or omission of CSL made little difference to the average benzaldehyde concentration in spent broth which was less than 0.100 gl^{-1} for all fermentations. The benzyl alcohol concentrations, however, ranged between 3 and 3.1 gl^{-1} for fermentations using 40% BBO and 60% raw sugar medium while the benzyl alcohol concentrations for fermentations from which CSL was omitted ranged from 2.5 - 2.7 gl^{-1}, a reduction of slightly over 10% (Table 4). Similarly to the benzaldehyde concentrations, the PAC yield achieved for the 40% BBO, 60% raw sugar medium was within the range of yields recorded for fermentations from which CSL had been omitted (11.2 - 14.9 gl^{-1}).

Given the similarity in biomass and PAC yields, similar q_{PAC} values were calculated for fermentations when 40% BBO and 60% raw sugar medium was used (0.0114 - 0.0145 $gg^{-1}h^{-1}$), regardless of the inclusion of CSL. These values were slightly higher than those recorded when 50% BBO and 50% raw sugar medium was used (0.0102 - 0.0110 $gg^{-1}h^{-1}$).

The average COD of spent fermentation medium from which CSL had been excluded (105,117 mgl^{-1}) was up to 10,000 mgl^{-1} lower than that of spent medium to which CSL had been added in some cases. These results suggested that despite the relatively small amount of CSL added to the growth phase medium, the effect on the organic load of the spent medium was considerable. The lower average COD was despite an increase in the reducing sugar concentration of the spent broth, in contrast to the previously positive correlation between the reducing sugar concentration and COD. The negative correlation may be due to the exclusion of non-carbohydrate organic compounds from the medium which would not be detected as variation in the reducing sugar concentration. The results demonstrated the impact of the addition of CSL to fermentation medium for limited benefit.

It was concluded that the inclusion of CSL in the medium offered limited benefit and it was therefore excluded from futher experiments. The omission of CSL enabled further simplification of the medium with reductions in nitrogen, metals, and other trace materials, all of which appear to be unnecessary to the success of the fermentation.

Effect of the omission of whey from fermentation medium

Using medium which contained 40% BBO and 60% raw sugar, and omitted CSL, the effect of whey on the fermentation was investigated. The omission of whey from the bioconversion phase medium resulted in an increase of approximately 15% in the pyruvate concentration early in the bioconversion phase, but this did not translate to an increase in the PAC yield, the yields being similar for all fermentations. The omission of whey did result in an approximate 5% reduction in the COD of spent broth, however no other effects were observed (Table 5).

TABLE 5 Concentrations of Dependent Variables at various Times during Fermentations with and without added Whey

	40 BBO:60 raw sugar-CSL	40 BBO:60 raw sugar-CSL-whey
Biomass (gl^{-1})	19.7	19.5
Pyrvate at acid rollover (gl^{-1})*	9.0	10.3
Final RS (gl^{-1})	65.7	53.55
Benzaldehyde (gl^{-1})	0.63	0.46
Benzyl alcohol (gl^{-1})	1.5	1.47
PAC (gl^{-1})	15.3	15.2
q_{PAC} ($gg^{-1}h^{-1}$)	0.019	0.020
COD (mgl^{-1})	110,950	105,200
PAC:benzyl alcohol	10.4	10.4

* Pyruvate sample collected at time when net pyruvate consumption equalled net production

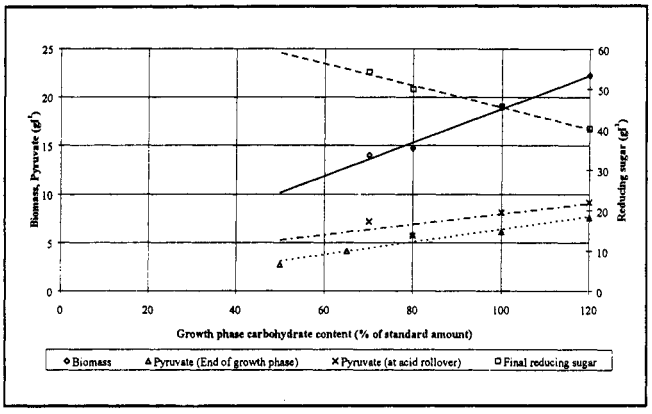

Fig.4 Effect of growth phase carbohydrate content on pyruvate, biomass and reducing sugar concentrations

TABLE 6 Length of the Growth Phase of Fermentations with varying Content of Carbohydrate-containing Materials in the Growth Phase Medium

Growth phase medium carbohydrate content (%)	Length of growth phase (h)
120	19.1
100	17.8
80	16.5
70	15.3
65	15.7
50	14.9

The whey may have originally been intended as a source of lactic acid for conversion to PAC, or as a source of thiamine, one of the co-factors of PDC, however, in this instance, no benefit was observed, its inclusion adding only to the cost of the medium and the COD of the spent broth. In keeping with the aim of this study to simplify the original medium formulation, whey was omitted from the bioconversion phase supplement in future fermentations.

Alteration of carbohydrate content in the growth phase of fermentations

Once the complex components of the medium were reduced or excluded, the total carbohydrate, nitrogen and phosphate contents were investigated with a view to further reducing their concentration. As for the complex components, carbohydrate content requirements were investigated independently of the requirements for nitrogen and phosphate. In turn, the growth phase carbohydrate requirement was examined separately from the bioconversion phase carbohydrate requirements.

During the investigation of carbohydrate requirements, the proportion of BBO, raw sugar and glucose syrup were all reduced equally, e.g., if a 20% reduction was made, all three components were reduced by 20%. The reduction of carbohydrate content in the growth phase resulted in a concomitant reduction in the pyruvate concentration measured at the end of the growth phase (Fig.4) as well as a reduction in the length of the growth phase (Table 6). If, as has been postulated (ref.11), that the PAC yield is limited by pyruvate concentration when pyruvate concentrations are low, then low yields might have been expected with low growth phase carbohydrate content.

The pyruvate concentration at the end of the growth phase ($p=0.003$), the final reducing sugar concentration ($p=0.031$) and the mid-bioconversion phase biomass concentration ($p=0.001$) were all clearly linked with the amount of carbohydrate present in the growth phase fermentation medium (Fig.4). The implication of the reduced intermediate pyruvate concentrations was that the net amount of pyruvate available

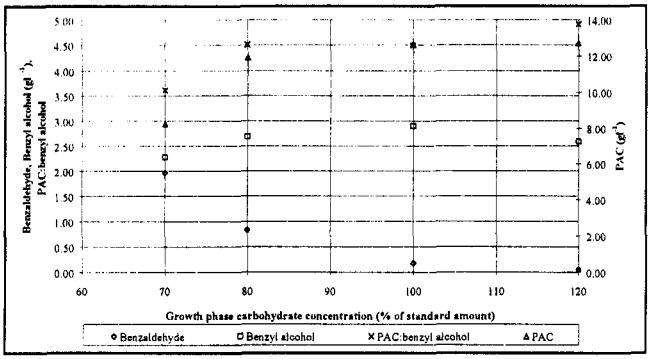

Fig. 5 Effect of gorwth phase carbohydrate on benzaldehyde, benzyl alcohol PAC concentrations, and PAC to benzyl alcohol ratio

for conversion to PAC was also reduced. The decrease in biomass concentration, with a reduced capacity for carbohydrate metabolism, was considered to be a contributing factor to the increased final reducing sugar concentration and may also have a reduced capacity for PAC production.

Residual benzaldehyde concentrations were significantly reduced as the growth phase total carbohydrate content was increased, and were counterbalanced by slight, although statistically insignificant, increases in PAC (approximately 4 gl^{-1}) and benzyl alcohol (1 gl^{-1}) concentrations (Fig.5). The changes in PAC and benzyl alcohol (and thus the PAC to benzyl alcohol ratio) did not fully account for the changes in benzaldehyde concentration. The decrease in residual benzaldehyde concentration, and increased PAC and benzyl alcohol concentrations with increasing carbohydrate content, was probably a result of the higher biomass concentrations and hence an increased rate of benzaldehyde metabolism. The increase in both benzyl alcohol and PAC concentrations with increasing carbohydrate content suggests that the reduction of carbohydrate-containing materials was detrimental, probably due to carbohydrate limitation.

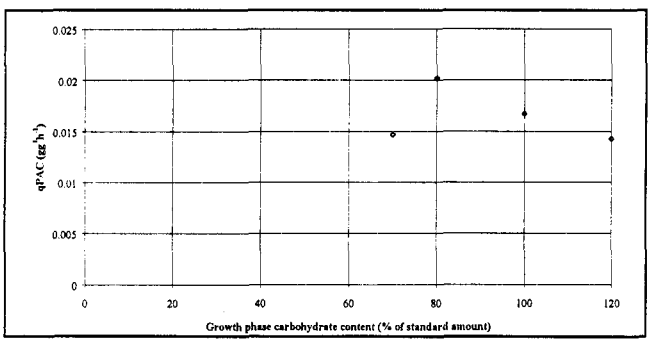

Fig. 6 Effect of growth phase carbohydrate content on the specific productivity of *C. utilis*

An increase in q_{PAC} was found with decreasing growth phase carbohydrate content until the latter was reduced to less than 80% of the standard concentration, when a large decrease in q_{PAC} was observed (Figure 6). The relationship between the carbohydrate content and q_{PAC} was extremely strong for carbohydrate concentrations between 80 and 120% of normal content ($p=1.57x10^{-5}$), thereby attributing virtually all of the change in productivity to the change in carbohydrate content.

Given the reliance of the pyruvate and PAC yields on the growth phase carbohydrate content, no change was made to the growth phase medium of subsequent fermentations.

Investigation of the effect of carbohydrate content during the bioconversion phase of PAC fermentations

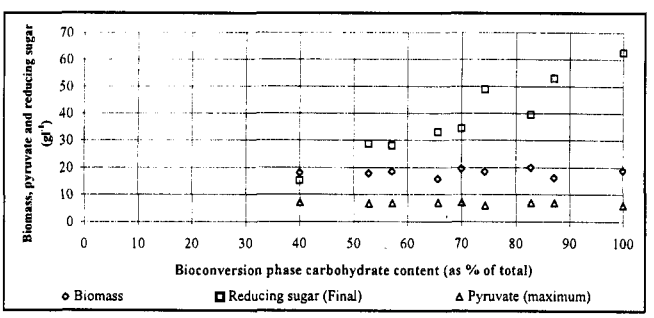

Fig.7 Effect of bioconversion phase carbohydrate content on biomass, reducing sugar and pyruvate concentrations

When the carbohydrate content of the bioconversion phase was altered, the carbohydrate contents of both the initial and second supplements were tested at three levels (100%, 70% and 40% of the original carbohydrate content) with each possible combination tested. Results, however, were analysed in terms of cumulative carbohydrate content from the lowest (40%) to the highest (100% of the original).

The biomass concentration was determined to be independent of the total amount of carbohydrate added during the bioconversion phase (p=0.695) (Fig.7) with the accumulation of additional biomass during the bioconversion phase of fermentations attributed to the probable accumulation of glycogen (ref.34). If glycogen accumulation was the principal cause of increases in biomass concentration, the mechanism used appeared to be concentration independent for the range of conditions tested.

Similarly to the biomass concentration, pyruvate yields appeared to be unrelated to the bioconversion phase carbohydrate content (Fig.7). Pyruvate excretion by *C. utilis* has been observed (ref.34) after conversion from glucose limitation to glucose excess, although there was no indication given of the concentration dependence of this phenomenon. It appears from the results here, that at the concentrations tested, there was no effect due to reducing sugar concentration. It is likely that the excretion of pyruvate is dependent on the conversion from carbohydrate limitation to excess, and on the relative rates of pyruvate production and consumption.

While biomass and pyruvate concentrations were independent of bioconversion carbohydrate content, the concentration of reducing sugar in the spent broth was strongly positively correlated ($p=8.2\times10^{-5}$). The average final reducing sugar concentration (17.3 gl^{-1}) for medium containing 40% of the standard carbohydrate content was equivalent to 25% of the average final reducing sugar concentration (56.8 gl^{-1}) in the standard fermentation medium (Fig.7).

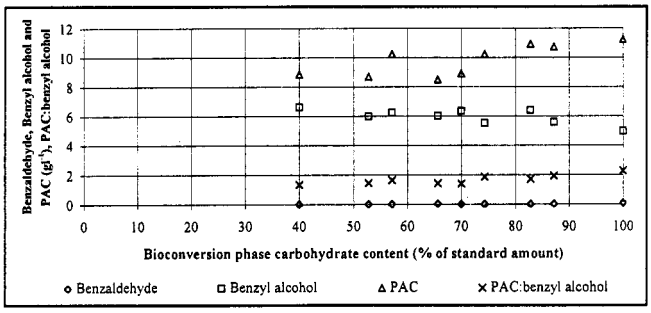

Fig.8 Effect of bioconversion phase carbohydrate content on the concentrations of benzaldehyde, benzyl alcohol and PAC, and on the PAC to benzyl alcohol ratio

Statistically significant variations in both benzyl alcohol and PAC yields were also measured. A 25% decrease in benzyl alcohol concentration (p=0.02) and a corresponding 25% increase in PAC yield (p=0.015) were recorded when the carbohydrate was increased from 40% of the standard fermentation to the standard medium composition (Fig.8). Increases or decreases in benzyl alcohol yield similar in magnitude but opposite in direction to the change in PAC yield have also been reported by other authors during their investigations of metabolic inhibitors and alternative hydrogen acceptors (refs.12,26,27,30). In the studies reported here, varying the carbohydrate content in the bioconversion phase supplement while maintaining a constant second supplement carbohydrate content had the greatest impact on PAC yield, with p values of 0.04 and 0.003 when fixed pump start carbohydrate contents of 40% and 100% (of the original content) were used respectively. These results suggest that nutrient supplementation immediately after the growth phase can influence the outcome of the fermentation.

Because the change in magnitude of PAC and benzyl alcohol concentrations were equal in size, but opposite in direction, there was an accompanying increase in the PAC to benzyl alcohol ratio from 1.3 to 2.2 and an increase in q_{PAC} of 20% (p=0.051) with increasing carbohydrate content. These ratios are considerably lower than previously obtained as a direct result of the higher than previously measured benzyl alcohol yields. The benefit of adding the standard amount of carbohydrate is clearly seen when the results are displayed in terms of conversion efficiency.

In conjunction with the lower final reducing sugar concentration as the level of added carbohydrate was reduced, there was a marked reduction in the COD of the extracted spent broth. When the total carbohydrate content of supplements was reduced to 40% of the standard amount, the COD was reduced from 112,700 mgl^{-1} for the standard fermentations to 70,000 mgl^{-1}, a reduction of almost 40%. The reduction of COD was very strongly linked with the amount of carbohydrate added to the medium ($p=2.73\times10^{-7}$) and was closely correlated with the residual reducing sugar concentration (p=0.0004). The reduction of BBO and raw sugar content in the medium therefore offers significant benefits in terms of reducing the organic load of the spent broth provided the PAC yield could be maintained.

A nominal concentration of 10 gl^{-1} PAC was set as the minimum required for process viability. The minimum required PAC concentration was not reliably achieved when the carbohydrate added during the bioconversion phase was less than 75% of the content in the standard medium. It is possible that the effect of the added carbohydrate was due to non-carbohydrate components which may stimulate PDC or physically buffer the biomass. Given the occasional variability of PAC yields between fermentations conducted at different times but using identical conditions, a higher carbohydrate content in the medium was ecommended. Therefore subsequent fermentations were conducted without modifications to the original carbohydrate content.

The effect of varying the nitrogen and phosphate content of growth and bioconversion phase medium

Nitrogen is essential for growth and metabolism, and by analogy with the known influences on metabolic activity conferred by carbohydrate sources, nitrogen sources can similarly affect metabolic activity, e.g., glycolytic activity can be controlled by nitrogen type and concentration (ref.3). The choice of nitrogen source has been demonstrated to influence fermentation yields, including PAC yields.

Phosphate is necessary for growth and glycolytic flux, is involved in controlling the synthesis of lipids and carbohydrates, helps to maintain cell wall integrity and is also influential in controlling the rate of fermentation (ref.3). Phosphate is a known allosteric inhibitor of PDC. Both *S. cerevisiae* and *C. utilis* PDC are competitively inhibited by phosphate with the PDC from *S. cerevisiae* being more sensitive to variation in phosphate concentration than *C. utilis* PDC (ref.34). Yeast requirements for phosphate are lower during fermentative metabolism than during respiratory metabolism, although acute phosphate deficiency will lead to reduced fermentative activity (ref.3).

After the modification of the original medium with respect to BBO content, and the omission of CSL and whey, the requirement of the yeast for nitrogen and phosphate was unknown. Based on the premise that reduction of the nitrogen and phosphate content in the fermentation medium would lead to a reduction in concentration in the spent medium and hence costly waste treatment, an investigation was undertaken to determine the effect of reducing the nitrogen and phosphate content in both the growth and bioconversion phase medium.

An arbitrary decision was made to reduce the content of nitrogen (as urea) and phosphate (as KH_2PO_4) by 15%. Both nutrients were therefore tested at two levels, the original (high) level and 85% of the original content (low level), for the growth phase and bioconversion phase supplements. Four factors were investigated (urea in the growth phase (N_g) and bioconversion phase (N_b) medium, and KH_2PO_4 in the growth phase (P_g) and bioconversion phase (P_b) medium). Each factor may cause an effect on a dependent variable independently of the other factors, i.e., a main effect, or may interact with a second factor to cause an effect, i.e., a two-factor interaction denoted as N_gN_b. A total of ten factors plus two-factor interactions were investigated. On examination of the data, possible trends were not immediately obvious so 'normal plots' were generated for each of the dependent variables monitored during the series of fermentations, e.g., PAC, by plotting the Normal Probability Score (the inverse of a normal cumulative distribution) against the Size of Effect (the difference in the average result for trials run at high and low levels). Effects which may be important, e.g., the effect of high growth phase urea and low growth phase phosphate concentration, appear as outliers on 'normal plots'. All of the outlying points for a given variable were analysed via linear regression to determine whether the apparent effect was statistically significant.

Initial examination of the normal plots indicated that the PAC yield, COD and reducing sugar concentration were affected by variation in the nitrogen and phosphate concentrations in growth and bioconversion phase media. From the normal plots, it appeared that PAC production was enhanced by high levels of phosphate and urea added at bioconversion, and low levels of urea present in the growth phase medium (Table 7). These requirements were commensurate with the predicted requirements for reduced COD and reducing sugar concentrations. On further analysis of the results (Table 8), however, none were found to cause statistically significant changes in the variables monitored. The PAC yield varied between 10 and 15 gl^{-1}, the COD fluctuated between 110,000 and 140,000 mgl^{-1} and the residual reducing sugar concentration between 40 and 60 gl^{-1}.

TABLE 7 Factors and Two-factor Interactions responsible for causing increases or decreases in the Response of Dependent Variables when varying the Concentrations of Urea and KH_2PO_4 in Fermentation Medium

Dependent Variable	Positive effect	Negative effect
Reducing sugar (prior to the initiation of the BP)	N_gP_b	-
Reducting sugar (AR)	N_gP_b	P_g
PAC	P_b, N_b	N_gN_b
COD	N_gP_B	-

TABLE 8 p-Values for Factors having Positive or Negative Effects on a Dependent Variable

	Reducing sugar (pre bioconversion) (gl^{-1})	Reducing sugar (acid rollover) (gl^{-1})	PAC (gl^{-1})	COD (mgl^{-1})
N_g	0.632	0.552	0.31	0.198
P_g		0.085		
N_b			0.287	
P_b	0.798	0.865	0.324	0.883
N_gN_b			0.273	
N_gP_b	0.586	0.375		0.583

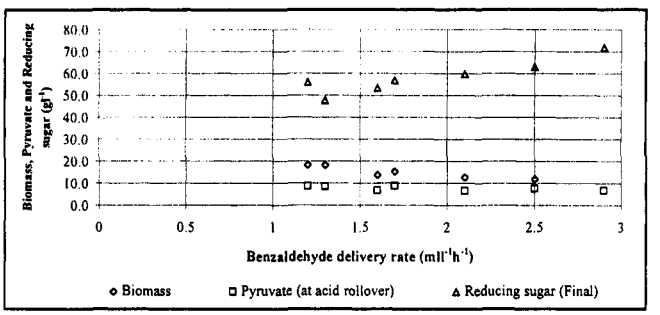

Fig. 9 Effect of benzaldehyde flow rate on biomass, pyruvate and reducing sugar concentrations

The lack of statistical significance suggested that there was a large amount of background noise and that the urea and KH_2PO_4 concentrations in both the growth and bioconversion phase media could be reduced by 15% with no significant reduction in PAC yield, or change in the COD. Relative to the COD attributable to carbohydrates, the COD due to urea is low which may explain why no reduction was noted in the COD of spent broth, however the reduction of both urea and phosphate concentrations would reduce the nutrient content of the spent broth. The results suggest that, although there was variation between the fermentations, the PAC production process is relatively robust and resistant to changes in medium composition.

The effect of benzaldehyde dosing rate on the production of PAC

As a minor aspect of this work, an investigation of the benzaldehyde flow rate, and its effect on the PAC fermentation process, was made. The toxic nature of benzaldehyde has previously been described in the introduction to this paper. A range of benzaldehyde flow rates from 1.2 $ml^{-1}h^{-1}$ to 2.9 $ml^{-1}h^{-1}$ were tested, with a number of effects being noted.

Increasing the benzaldehyde flow rate reduced the pyruvate concentration by approximately 1 gl^{-1}, the biomass concentration by approximately 5 gl^{-1} (Fig.9) and appears to be consistent with the known toxic nature of the benzaldehyde. As a result of the reduction in biomass concentration, and probably also a result of impaired glycolysis, there was an increase in the concentration of residual reducing sugar of almost 10 gl^{-1} as the benzaldehyde delivery rate was increased. The benzyl alcohol yield was also reduced by approximately 50% and was probably the result of earlier inactivation of ADH by residual benzaldehyde under conditions of high benzaldehyde flow. Slight increases in the PAC yield (1 - 2 gl^{-1}) were measured while the residual benzaldehyde concentration was increased by up to 100% (Fig.10).

In this instance, the production of PAC appeared to be independent of pyruvate concentration. Therefore the beneficial aspects of higher benzaldehyde flow, namely increased PAC to benzyl alcohol ratio could be exploited. There were also increases in q_{PAC} which corresponded with the increase in PAC and the larger decrease in biomass concentration.

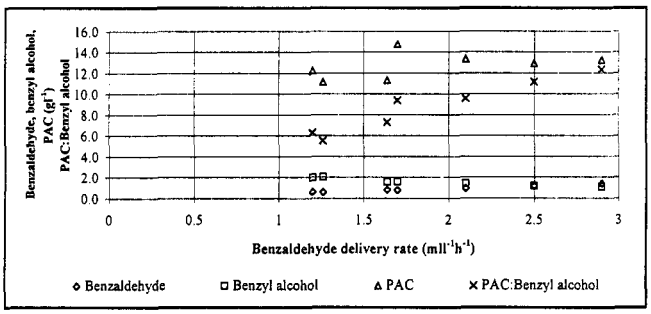

Fig. 10 Effect of benzaldehyde delivery rate on the concentrations of benzaldehyde, benzyl alcohol and PAC, and on the PAC to benzyl alchol ratio

CONCLUSIONS

The original medium used by ICI in their industrial fermentation process for the production of PAC contained many components which were not necessary for the maintenance of PAC yields and which, in many cases had a detrimental effect on the PAC to benzyl alcohol ratio, q_{PAC} and the COD of the spent fermentation broth. Substantial increases in the performance of the fermentation could be achieved by simplifying the industrially used fermentation broth with potential cost benefits in the reduction of material, transport and storage costs, and later, reduction in the costs associated with down stream processing and waste treatment.

REFERENCES

1. Zabriskie, D.W., Armiger, W.B., Phillips, D.G. and Albano, P.A. (1980) *Traders' Guide to Fermentation Media Formulation*. Traders' Protein Division, Traders' Oil Mill Company.
2. Oura, E. (1983) Chapter 1a: Biomass. *In* Rehm, H-J. and Reed, G. (Eds.) *Biotechnology: A comprehensive treatise*. (Volume 3) Verlag-Chemie, Weinheim, Germany
3. Jones, R. P. and Greenfield, P. F. (1984) A review of yeast ionic nutrition. Part I: growth and fermentation requirements. *Process Biochemistry* 19(2):48-60
4. Cejka, A. (1985) Ch 26: Preparation of Media. pp 630-698 *In* Rehm, H-J. and Reed, G. (Eds.) *Biotechnology: A comprehensive treatise (Volume 2)* Verlag-Chemie, Weinheim, Germany.
5. Reed, G. and Peppler, H. J. (1973) *Yeast Technology* AVI, Connecticut, U. S. A.
6. Meyrath, J. and Bayer, K. (1979) Biomass from whey. *In* Rose, A. H. *Economic Microbiology* Vol 4 pp 207-269 Academic Press, London.
7. Berry, D. R. and Brown, C. (1987) Physiology of yeast growth. pp159-199 *In* Berry, D. R., Russell, I. and Stewart, G. G (Eds.), *Yeast Biotechnology* Allen and Unwin, London.
8. Culik, K., Netrval, J., Souhrada, J., Ulbrecht, S., Vojtisek, V., Vodnansky, M. (1984) Czech patent No. 222 941
9. Stanbury, P. F. and Whitaker, A. (1984) *Principles of Fermentation Technology*. pp 74-90 Pergamon Press, Oxford, Great Britain.
10. Dissara, Y. and Rogers, P. L. (1995) Evaluation of mutants of *Candida utilis* for L-PAC production from benzaldehyde. *4th Pacific Rim Biotechnology Conference* pp 248-249
11. Vojtisek, V. and Netrval, J. (1982) Effect of pyruvate decarboxylase activity and of pyruvate concentration on the production of 1-hydroxy-1-phenylpropanone in *Saccharomyces carlsbergensis*. *Folia Microbiologica* 27:173-177
12. Rogers, P. L. (1990) ICI Internal Report
13. Noronha, S. and Moreira, A. R., (1993). Bioconversion of benzaldehyde by yeast. *Abstracts of Papers American Chemical Society* 205(1-2) Biot. 173
14. Noronha, S. (1996) Personal communication.
15. Agarwal, S. C., Basu, S. K., Vora, V. C., Mason, J. R. and Pirt, S. J. (1987) Studies on the production of L-acetyl phenyl carbinol by yeast employing benzaldehyde as precursor. *Biotechnology and Bioengineering* 29:783-785
16. Tripathi, C. K. M., Basu, S. K., Vora, V. C., Mason, J. R. and Pirt, S. J. (1988) Continuous cultivation of a yeast strain form biotransformation of L-acetyl phenyl carbinol (L-PAC) from benzaldehyde. *Biotechnology Letters* 10(9):635-636.
17. Ellaiah, P. and Krishna, K. T. (1987) Studies on the production of phenyl acetyl carbinol from benzaldehyde by *Saccharomyces cerevisiae*. *Indian Drugs* 24(4):192-195
18. Ellaiah, P. and Krishna, K. T. (1988) Effect of aeration and alternating current on the production of phenyl acetyl carbinol by *Saccharomyces cerevisiae*. *Indian Journal of Technology* 26:509-510
19. Nikolova, P. and Ward, O. P. (1991) Production of L-phenylacetyl carbinol by biotransformation: product and by-product formation and activities of the key enzymes in wild-type and ADH isoenzyme mutants of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* 20:493-498
20. Long,A. and Ward, O. P. (1989) Biotransformation of aromatic aldehydes by *Saccharomyces cerevisiae*: investigation of reaction rates. *Journal of Industrial Microbiology* 4:49-53
21. Long, A. and Ward, O. P. (1989). Biotransformation of benzaldehyde by *Saccharomyces cerevisiae*: Characterization of the fermentation and toxicity effects of substrates and products. *Biotechnology and Bioengineering* 34:933-941
22. Becvarova, H. and Hanc, O. (1963) Production of phenylacetylcarbinol by various yeast species. *Folia Microbiologica* 8:42-47.

23. Voets, J. P., Vandamme, E. J. and Vlerick, C. (1973) Some aspects of the phenylacetyl carbinol biosynthesis by *Saccharomyces cerevisiae*. *Zeitschrift feur Allgemeine Mikrobiologie* 13:355
24. Long, A., James, P. and Ward, O. P. (1989) Aromatic aldehydes as substrates for yeast and yeast alcohol dehydrogenase. *Biotechnology and Bioengineering* 33:657-660
25. Mahmoud, W.M., El-Sayed, A. H. and Coughlin, R. W. (1990b) Production of L-phenylacetyl carbinol by immobilised yeast cells: II. Semicontinuous fermentation. *Biotechnology and Bioengineering* 36:55-63
26. Smith, P. F. and Hendlin, D. (1953) Mechanism of phenylacetylcarbinol synthesis by yeast. *Journal of Bacteriology* 65:440 - 445
27. Ose, S. and Hironaka, J. (1957) Studies on production of phenyl acetyl carbinol by fermentation. *Proceedings of the International Symposium on Enzyme Chemistry* 2:457-460
28. Mahmoud, W. M., El-Sayed, A. H. and Coughlin, R. W. (1990a) Production of L-phenylacetyl carbinol by immobilised yeast cells: I. Batch fermentation. *Biotechnology and Bioengineering* 36:47-54
29. Mahmoud, W. M., El-Sayed, A. H. and Coughlin, R. W. (1990c) Effect of β-cyclodextrin on production of L-phenylacetyl carbinol by immobilised cells of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* 36:256-262
30. Smith, P. F. and Hendlin, D. (1954) Further studies on phenylacetylcarbinol synthesis by yeast. *Applied Microbiology* 2:294
31. Thompson, A. G. (1988) Industrial waste management: an overview of recent progress. *Chemeca* pp 66-73
32. Hach Company (1992) *DR/2000 Spectrophotometer Procedures Manual.*
33. Haaland, P. D. (1989) *Experimental Design in Biotechnology* Marcel Dekker, Inc., New York.
34. van Urk, H., Mak, P. R., Scheffers, W. A. and van Dijken, J. P. (1988) Metabolic responses of *Saccharomyces cerevisiae* CBS 8066 and *Candida utilis* CBS 621 upon transition from glucose limitation to glucose excess. *Yeast* 4: 283-291