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Technical report: Molasses Fermentations

Prepared for the Government of India
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Molasses Fermentations

an ab initio analysis

Primary objectives of the NCL programme

To construct and operate a demonstration unit with an economically significant performance, for the conversion of molasses sugar into ethanol, suitable for operation in conjunction with NCL heat-pump-assisted distillation technology.

In order of priority which takes economic costs of practical ethanol manufacture into account, the consequent specific requirements are:
1. A high conversion of the input fermentable sugar, supplied as molasses (i.e. better than 95%) to a good yield (≥ 95%) of ethanol at a concentration which can be economically distilled (≥ 5% w/w).
2. A reliable process that can be run at low operating cost.
3. Operational simplicity.
4. Minimal capital cost consistent with 1-3.

The available background work within NCL comprises:
1. Reasonable success in preparing yeast in various immobilized particulate forms whose properties are reasonably established.
2. Experience with continuous reactors at various scales using the immobilized yeasts with molasses and other substrates.
Thus there is an *a priori* preference for a continuous reactor using an immobilized yeast.

Nevertheless the problem, having now reached the final stages of the project itself, requires re-examination from first principles in order to confirm the primary objective and its consequent requirements. In the following *ab initio* analysis it is shown that optimal utilisation of the immobilized yeast approach requires well-mixed reactor conditions, despite the more obvious theoretical advantages of plug flow operation. Optimal running can then be obtained *either* by cyclic re-use of fed-batch reactors *or* by continuous operation of a series of reactors with split substrate feeds and regular reactor sequence switching. In addition however it is also shown that when certain physical properties of the immobilizates are taken into account, very practical alternatives can be devised, corresponding essentially to both these possibilities, using an "auto-immobilizing" (i.e. permanently flocculated) yeast.

Defining the process profile

With a molasses substrate, containing both fermentable sugars (FS) and inhibitory molasses salts (MS) and yielding ethanol (P) from the FS the overall process profile can be represented as:

$$\left.\begin{matrix} FS \\ MS \\ H_2O \end{matrix}\right\} \rightarrow \left.\begin{matrix} P \\ MS \\ H_2O \end{matrix}\right\}$$

In a pure batch process this profile is followed in time; in a pure continuous plug flow process it is followed in space; obviously intermediate conditions where the profile is followed partly in time and partly in space are feasible.

Note that the amount of MS is strictly proportional to the FS that has been supplied up to any point in the profile, and to the P formed provided that the yield, $Y_{P/FS}$, has been maintained. We shall take $Y_{P/FS}$ as 0.45-0.47. It is required that P/H_2O shall be over 5% and for the present illustrative calculations we shall set it as 65 g/L. Hence FS/H_2O will be $65 / Y_{P/FS} \equiv 140$ g/L. To have a numerical value for MS we shall set it at FS = 10MS; thus in the overall process profile above, MS is 14 g/L throughout.

The catalyst

For an intensified process it must be possible in one way or another to have a significantly longer residence time for the catalyst than for the aqueous components. This is achieved using the physical characteristics of the catalyst. Using an immobilized yeast, the most relevant physical characteristics are the friability and the effective density. Similar characteristics can be considered for autoimmobilized yeast particles.

Particles prepared from good quality alginate have been shown to have satisfactory friability provided shear forces are kept low, as in a fixed or partly-fluidized bed. Their friability in a well-mixed but low-shear regime, such as can be obtained using a gas-lift effect for bulk mixing, will probably be satisfactory, but they can not be directly exposed to mechanical mixing devices such as turbine impellors. Particles of auto-immobilizing yeast have similar properties, with the important difference that a detached fragment formed through any friability has a good chance of regenerating a new particle.

Particles prepared from alginate have under most conditions a marked buoyancy. This is particularly true at high FS concentrations and when CO_2 is being actively evolved; when the substrate is fully "attenuated" (i.e. FS *ca* 0) and no gas is forming, the particles will sink. Particles of auto-immobilizing yeasts have considerable negative buoyancy under most conditions and de-gas rather readily; their "stickiness" when in contact and at rest is however appreciable.

Beyond this, however, we need to consider the biological properties of the catalyst, which are essential for the process and which are a property of the yeast *as subjected to the process profile.*

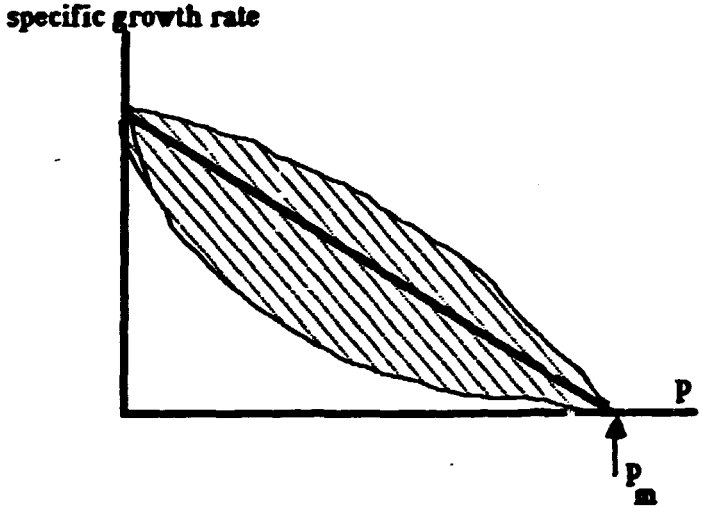
Yeast properties in relation to the process profile

However the process is arranged, unless there is a system for removing ethanol concurrently with its formation, it is inevitable that the yeast is, at some stage at least, exposed to the final conditions, which we have taken for our system as being P - 65 g/L, MS - 14g/L. Along preceding stages in the process profile we can also say that under all conditions P is increasing up to this value and that MS is either increasing or has its final value throughout.

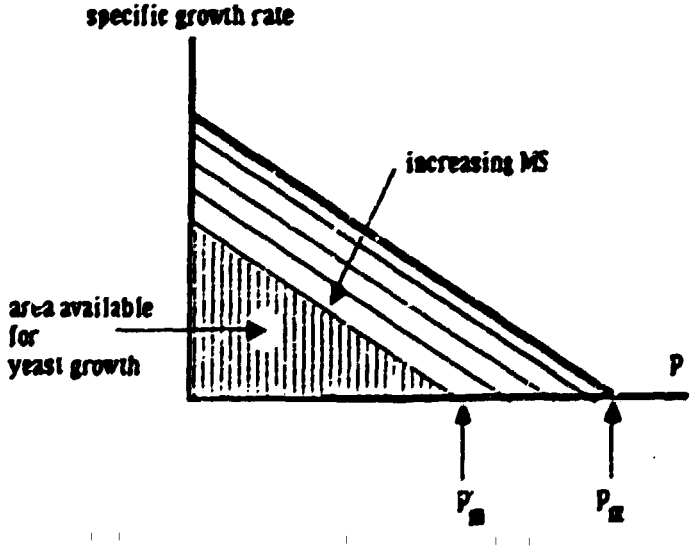
Under these conditions the catalytic properties of the yeast are severely inhibited. More critically, since the yeast must always, to at least some extent, replace itself by growth, it must be capable *either* of some growth at the final conditions *or else* the system must permit renewed growth at some subsequent less severe condition. The variation of yeast growth rate with ethanol concentration P, and hence along the process profile, can be represented as in the annexed figure, which ignores the effects of other factors on the growth rate.

Effect of ethanol on yeast growth:

The precise shape of the curve is not material and in later versions of this figure only the straight line plot is shown.The point P_m will be very close to the operating value chosen for $P_{(final)}$

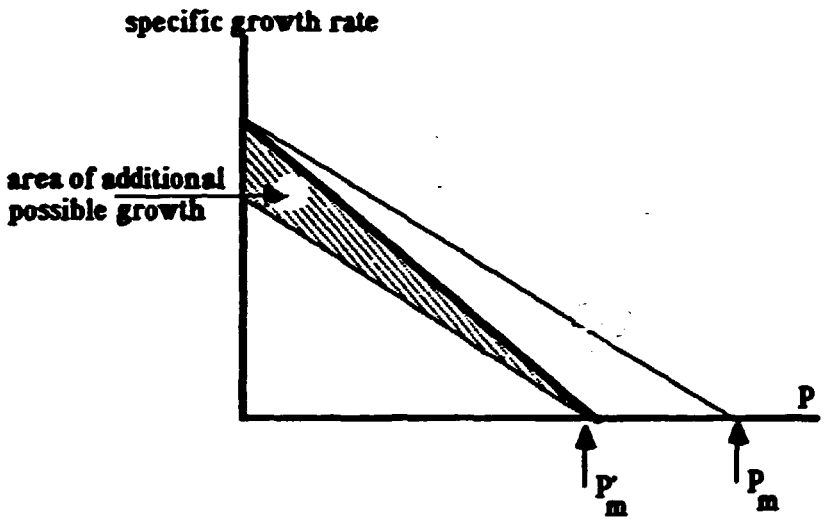


Now the effect of the further inhibition by molasses salts MS is most simply considered as lowering both μ_m and P_m, as shown in the second figure:



In effect, then, the whole curve is shifted as shown; not only are the values of the growth rate lower, so also is the maximum level of ethanol permitting growth, P'_m.

The area underneath the curve of μ *versus* P represents the totality of *available* growth; it can be maximised by making MS proportional to P rather than, as assumed so far, making it constant throughout - that is, by only adding (FS+MS) in amounts sufficient to sustain the increase in P, for example in a "fed-batch" mode. The change in the μ *versus* P plot is then as in the third figure:



This gives the additional growth possibility shown by the shaded area.

From all three versions of our μ *versus* P plot it follows that the best conditions for yeast growth are those at the initial stage of the process profile.

Now it is an important but complicating factor that, whereas we have so far considered the effects of P and MS on growth as "instantaneous" in nature, in reality they are time-dependent. The longer the time spent by the yeast in conditions, for example, where P approaches P_m, the more severely it is affected. Yet it is also necessary for the yeast to reach precisely this condition, as we have seen.

Hence for an optimal continuous or quasi-continuous system the yeast must *either* be returned to the initial conditions as soon as possible, *or else* it must be replaced by yeast which has hitherto been in those conditions. This is done, for example:

(1) in repeated batch systems when the yeast is re-used (Melle-Boinot &c)

(2) in a multi-stage continuous system in which there is some "forward" transfer of yeast (UMAPP-II system).

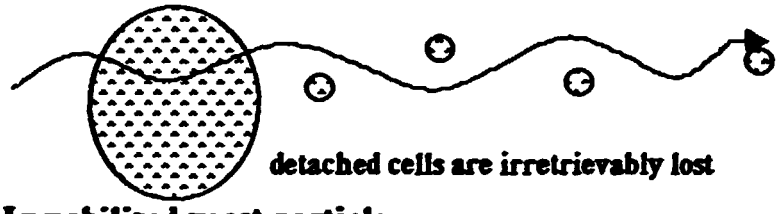
It can *not* be done in:

(1) a homogeneous continuous reactor (HCR) operated too close to $P=P_m$

(2) a pure plug-flow fixed-bed reactor.

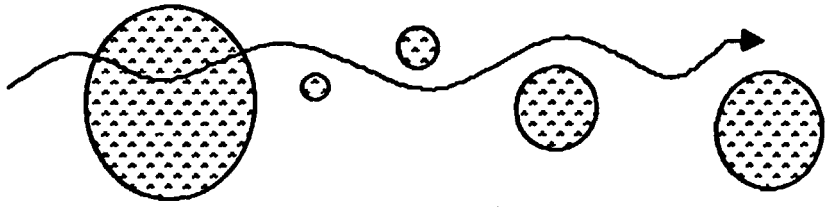
With a true HCR system stable operation can only be *sustained* if $P_{(final)}$ is significantly less than P_m; in addition to the effects of inhibitors, yeasts have a "natural" death rate of, say, one cell per five or six divisions, that is 1 in 2^{5-6} or about 1-2%; thus the maximum *usable* level for $P_{(final)}$ is about $0.9.P_m$.

Correspondingly, a fixed-bed "plug-flow" reactor can actually be maintained at P_m or indeed higher *provided that* there is actual physical movement of at least some cells along the reaction path, that is, into high-inhibition conditions from low-inhibition conditions. In considering this possibility, however, there is an important difference between conventional immobilized yeast and an auto-immobilizing (permanently flocculating) yeast, and this is illustrated in the figure:



detached cells are irretrievably lost

Immobilized yeast particle



detached cells generate new particles

Auto-immobilizing yeast particle

This effect adds cell loss by detachment to the effects of growth inhibition in a reactor using entrapped yeast particles. On the other hand cell detachment in a reactor using yeast which is simply attached by "natural" adhesion to an inert support can be the mechanism providing the element of forward flow which we have seen is a desirable feature.

Contamination of the catalyst

Contamination of the catalyst by unwanted and deleterious micro-organisms is an important practical aspect that is usefully considered at this point. Given that the ability of yeasts to survive in relatively high concentrations of ethanol is not shared by many other organisms, significant contaminants in an ethanol-producing reactor are confined to relatively special conditions. However, the following considerations may be important:

(1) Film-forming or similarly 'adhesive' bacteria that can survive in other respects may be encouraged by the use of a non-renewing particulate catalysr as well as by 'dead spots' in the flow regime.
(2) Persistent exposure to high osmotic pressure (locally high FS) in feed lines or inlet points or at the beginning of a plug flow reactor will encourage the growth of osmophilic contaminants.
(3) Contamination can be reduced by inhibitors (bisulphite, antibacterials, low pH).
(4) Non-adhesive contaminants will be eliminated at high fluid throughput rates.
(5) Contamination will be minimal at high levels of ethanol.

Thus consideration of contamination problems can lead to conclusions which are superficially opposite to those reached by considering inhibition problems:

inhibition effects indicate that the yeast should for a significant part of its total reactor residence time be exposed to minimal levels of ethanol and FS.

contamination effects indicate that the yeast should similarly be exposed for significant and recurrent periods to high ethanol levels.

Interim conclusions for yeast selection criteria

Already it is possible to define certain criteria for yeast selection which are additional to such obvious desiderata as high ethanol yield, rapid fermentation and good temperature tolerance. Yeast selection must be relevant to the actual process conditions. In particular: –

(1) The yeast must be selected under essentially anaerobic conditions on a molasses substrate, experiencing the combined effects of FS, MS, and P.

(2) The yeast must be selected in relation to it's performance under the whole range of FS/MS/P values it will encounter in the process profile *and in relation to its pasing through that profile many times.* Thus no single set of conditions will of itself provide the requisite selection pressure.

(3) Single-set conditions - for example in a steady-state HCR - can be used to select for specific characteristics but the selection must be checked continuously against the full range of process conditions to ensure that other essential characteristics are not being lost. For example simple selection for growth at high FS+MS will result in the selection of unwanted osmophiles with poor P yields.

(4) A steady-state HCR is intrinsically unstable, in practice justifiable on theoretical grounds, at dilution rates close to μ_m when operated in the usual chemostat mode, becoming extremely sensitive to inhomogeneities and highly selective for cells or colonies which are selectively retained, e.g. through adhesion or flocculation. For stable operation in this region, when the effective value of μ_m is to be determined by inhibitor effects, the HCR must be operated in the turbidostat mode.

In its usual form the turbidostat cannot be used for yeast growing on molasses. However, an equivalent "trophostat" mode can be devised and might be used. For a fermenting yeast at P levels approaching P_m, the specific rate of ethanol production remains non-zero though dependent like the growth rate on P. The *volumetric* rate, and also the volumetric rate of CO_2 evolution, also depends upon

the yeast concentration x, so that provided P does not vary greatly either the rate of CO_2 evolution or the effluent ethanol concentration can be used as indirect measures of x, and can therefore be used as control measurements to regulate either the dilution rate or the inputs of substrate and/or inhibitors. Such a system will exert effective selection pressure for performance near $P_{(final)}$.

A shorter-term selection procedure which might be useful if applied with care and caution would be to adapt a yeast growing at 90% of μ_m on *ca* 90 g/L FS supplemented with disproportionally-increasing levels of MS obtained from stillage, using a conventional chemostat operated as closely as possible to truly *homogeneous* and *steady* conditions (good mixing, accurate regulation of D).

Some Possible Process Arrangements.

We shall now consider some process arrangements which, it is suggested, are the most appropriate in terms of the preceding analysis and also are the most opportune for the NCL programme; that is, they conform as closely as possible to the primary objectives and consequent requirements, they make the maximum use of the varied experience already aquired by the NCL teams, and they also conform to the further requirements that emerge from our *ab initio* analysis, while offering reasonable prospects of success on a relatively short time-scale.

The process arrangements that will be considered are:

(1) A three-stage continuous process with three similar well-mixed reactors working heterogeneously in series, with a divided substrate feed, with regular rotation of reactors between process stages.

(2) A fed-batch process in a well-mixed reactor with re-use of the catalyst for successive batches (ideally two or more such reactors would be running in parallel an a phased manner).

(3) A continuous well-mixed reactor working heterogeneously and feeding outputs of both catalyst and weak "beer" to successive batch reactors with additional substrate feeds.

All three of these modes can be considered using either yeast entrapped in artifical particles, e.g. of alginate, or an auto-immobilizing (permanently-flocculated) yeast as the catalyst. However a preference for the latter will be developed.

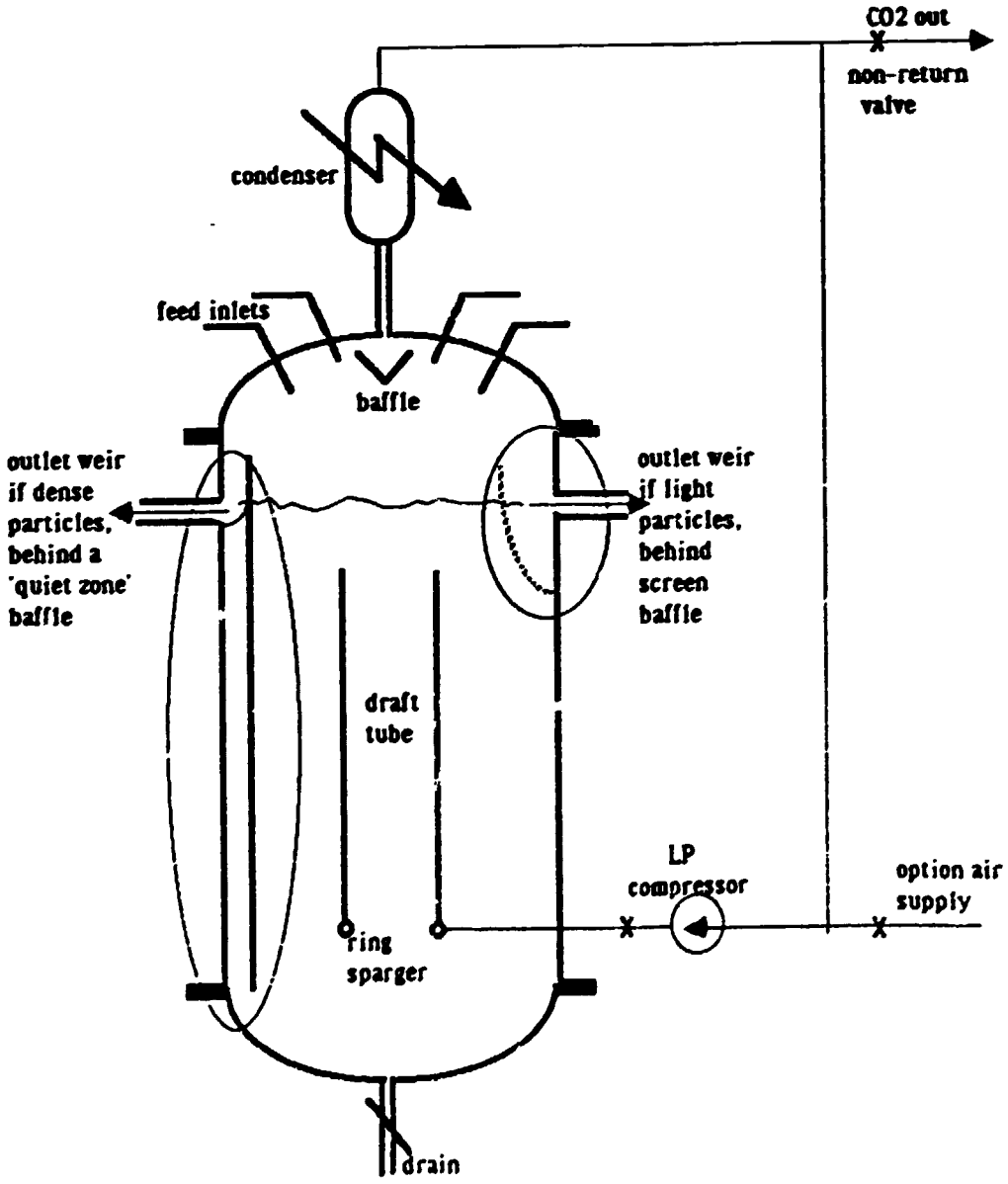
The reactor design for all three modes is effectively the same in that it avoids locally deleterious conditions by ensuring good bulk mixing of the fluid phase, using the gas-lift effect produced by recycling part of the process CO_2 into a draft-tube or similar arrangement so as to produce fluid velocities sufficient to maintain free circulation of the suspended particles.

A possible arrangement for such a reactor is shown below, with some comments on dimensions etc. in the following figure. The diagram shows two possible arrangements of the outlet system, depending on whether the catalyst particles tend to float or to sink.

For particles which tend to float, e.g. an alginate immobilizate, the requirements are (1) to maximise fluid velocities in the downcomer, and (2) to separate particles from the outflow by a suitable screen. For particles which tend to sink, e.g. an auto-immobilizing yeast, the outflow is to be taken from the top of a "quiet" zone which opens into the bottom of the reactor allowing the particles to settle out below the exit weir.

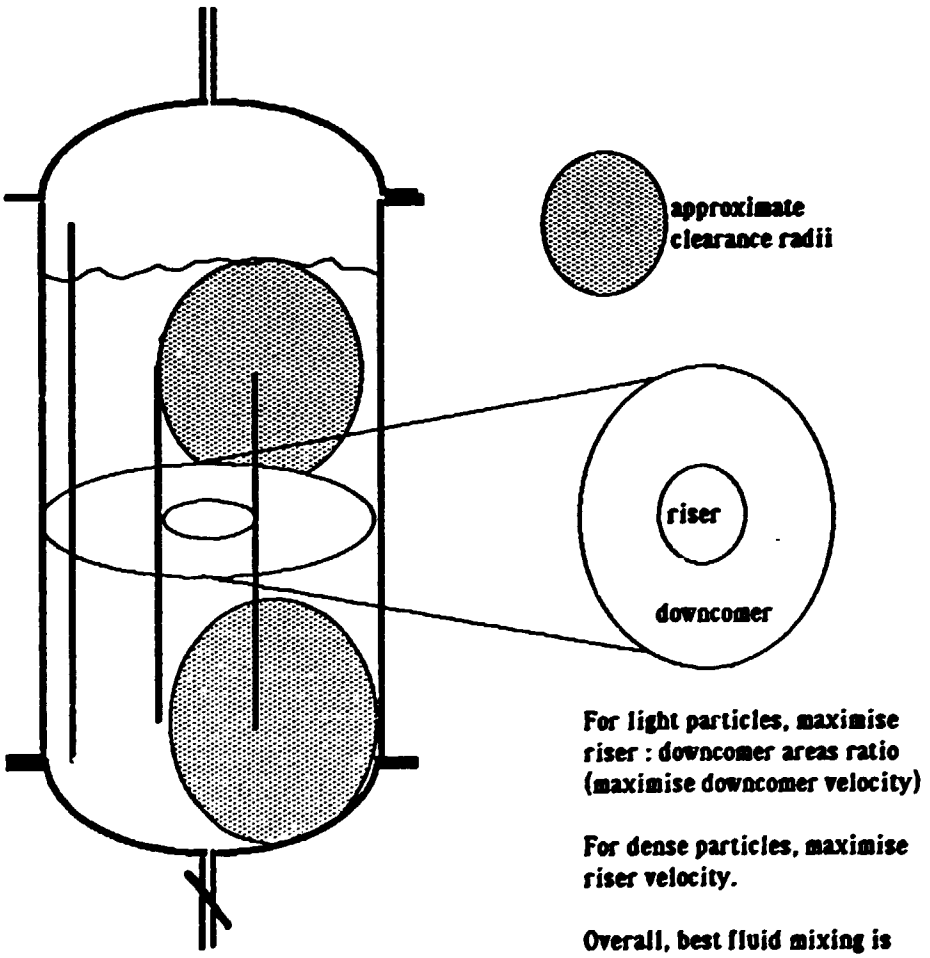
Arrangements for temperature control etc. are omitted for clarity; because of the aspect ratio and flow patterns it will normally be sufficient to provide heat exchange through the outer vessel wall.

General reactor layout



Note that some alternative arrangements were discussed in detail with NCL staff; these involved securing adequate fluid velocities in the reactor by means ofexternal pumps, or by using a shrouded impellor inside the reactor. It was decided that the capital and running costs of these arrangements were likely to be prohibitive.

Clearance radii etc. in the reactor



For light particles, maximise riser : downcomer areas ratio (maximise downcomer velocity)

For dense particles, maximise riser velocity.

Overall, best fluid mixing is when riser and downcomer areas are about equal.

A suitable aspect ratio would be 3:1 or greater (external, on the working volume). Suitable working volume (pilot scale) 100 L.

Reactors of the above design have been successfully scaled up to 0.1 m^3 and work well with an auto-immibilizing yeast, sustaining in free suspension up to 100 g dry wt. of catalyst per L.

One problem, however, is that with an immobilized yeast catalyst (e.g. alginate particles) the corresponding particle density gives an appreciably lower concentration of the yeast itself, and consequently a lower limit to the specific production rate per unit volume of the reactor. An auto-immobilizing yeast particle is 100% yeast, and even allowing for a lower specific production rate due to

diffusion effects, its effectiveness per particle is considerably greater than that of a largely inert catalyst particle.

Alternative process systems using this general type of reactor will now be discussed, in order of increasing complexity. However some preliminary information will enable the key decision, about whether to use an immobilized yeast or an autoimmobilizing yeast as the catalyst, to be made at an early stage, and this is clearly desirable.

Preliminary experimentation.

After constructing a simple gas-lift-effect mixed reactor, simple tests - in which compressed air can briefly be used as the sparging gas, will determine whether the alginate-bead immobilizate can be fully suspended by this means, with a bead volume of up to 30%. The test should be done with beads which are actively fermenting and evolving CO_2 freely; a suitable medium would be molasses at 8%FS. Satisfactory suspension in this case will involve maximising the fluid velocity in the downcomer; for maximum efect, the sparging gas needs to be well-dispersed since the mixing effect depends on gas hold-up in the riser. Since the immediate objectives are severely practical, namely to construct a working system which can be demonstrated and which can be scaled up in a realistic way, *the results of this experiment should determine the decision whether to persist with developments using the immobilized yeast or to proceed directly with work on an auto-immobilizing (i.e. permanently flocculated) yeast.*

Fed-batch operation.

After construction and simple hydraulic testing the reactor should be run in a fed-batch mode. This is best approached semi-empirically. If an auto-immobilizing yeast is used, an adequate yeast population must be accumulated in the reactor; this is best done by running continuously, with air as the sparge gas, at a high dilution rate (at least $D = 1\ h^{-1}$) and with a dilute feed (not more than 10 g/L FS, initially as jaggary, subsequently as molasses. When an adequate yeast population has been built up (at least 50 g dw/L), or when the reactor has been loaded with the maximum useful density of immobilized yeast particles, concentrated molasses should be fed (with minimum diluting water) to an initial FS content of 50 g/L. Feeding with undiluted molasses will reduce contamination possibilities in feed-lines as well as avoiding an excessive volume increase in the reactor. When gas evolution slows down, or when analysis shows FS below 5 g/L, further molasses should be fed at an appropriate rate so that the fed batch can be stopped when, say, a total of 110 g/L FS has been fermented.

The reactor should then be drained. The reaction should be stopped before the maximum possible amount of alcohol has been formed, so that the yeast is not exposed to excessive inhibition. It may be possible to flush it to remove accumulated molasses fines. To encourage re-growth, the succeeding batch should again be started under mild conditions - a brief period of aerobic growth on dilute feed at high dilution.

The advantage of this system is that it combines fully efficient re-use of the yeast (avoiding both the time and the substrate required for yeast growth in each batch) with a high-intensity fermentation (since the re-used yeast population is considerably higher than in normal batch fermentations) with the advantages - in terms of lower inhibitor levels through most of the batch - of fed-batch operation.

To install this system on a large scale would of course imply installation of the improved reactor (etc.), plus the immobilization equipment unless an auto-immobilizing yeast is used.

Continuous culture, optionally with batch "finishing"

A second mode of operation can now be tried, using the same reactor in continuous mode.

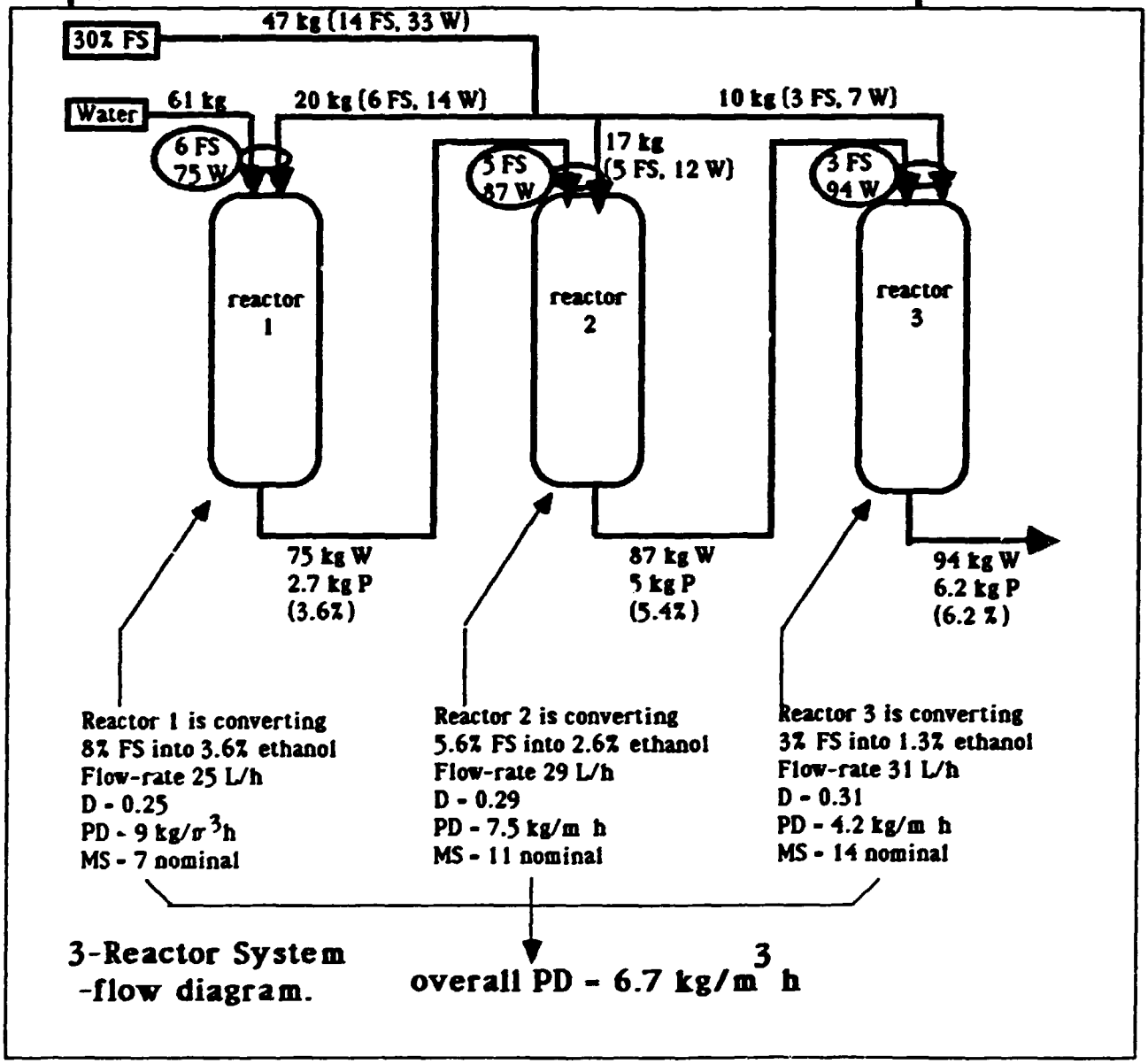
The reactor is fed with a steady input of about 300 g/L FS; again, separate feeding of clean water and concentrated molasses is desirable to minimise contamination. The dilution rate should be raised sufficiently to give about 40 g/L ethanol in the outflow (probably D = 0.5 h^{-1} or higher) leaving about 10 g/L excess FS. The outflow is collected in batches and fermented further with added molasses and a conventional yeast system, up to (say) 60g/L ethanol; the yeast from this operation will probably not be suitable for re-use.

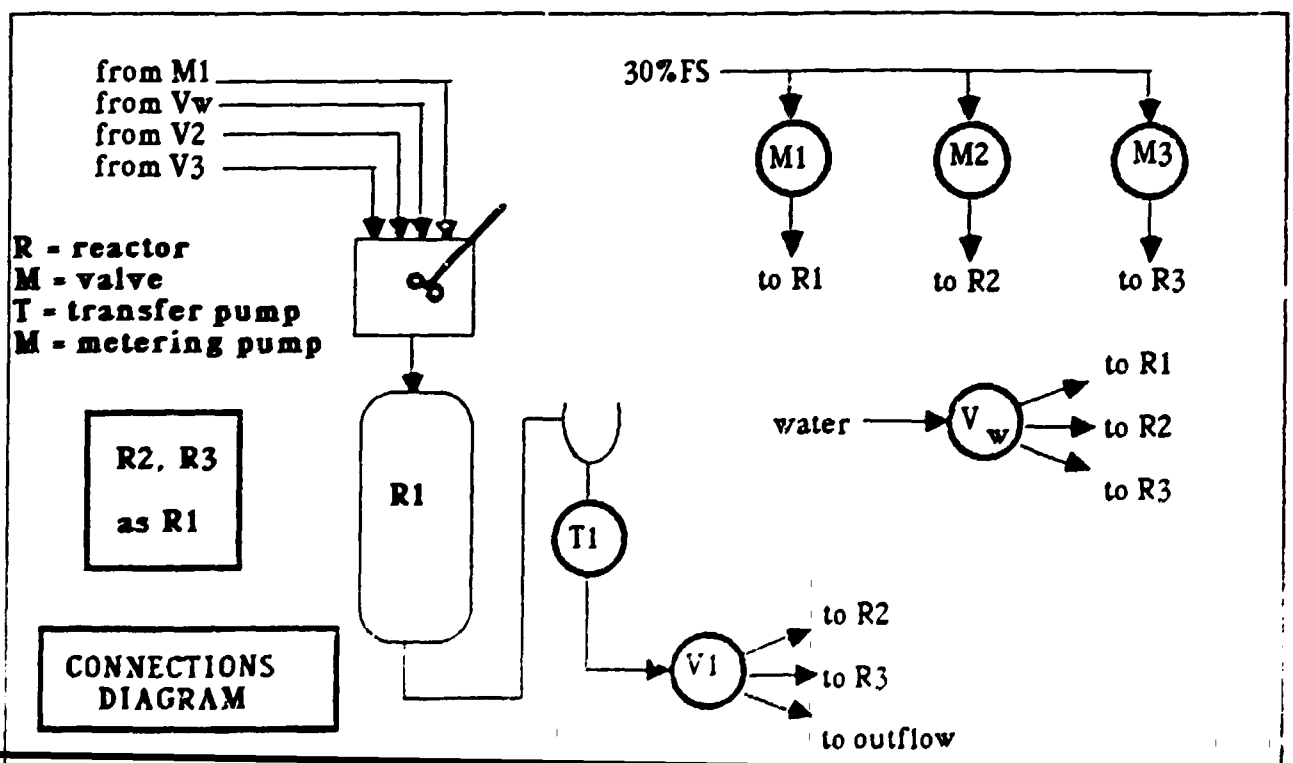
The advantage of this mode of operation is, effectively, in increasing the volumetric productivity for the first part of each batch, without sacrificing the potential of batch systems for reaching higher final ethanol levels.

Its full-scale installation in an existing distillery would require the fitting of one continuous reactor, essentially as above, serving a battery of batch reactors to be used for "finishing".

When testing this concept, it is a simple extension to test the continuous system to progressively higher final ethanol levels, *provided that as the final ethanol level is raised the yeast is repeatedly tested for its ability to "recover" with reasonable rapidity when subsequently exposed to low ethanol levels.* An alternative way to reach the same assessment is simply to ensure that the system is really stable for long-term running at the selected final ethanol level. However, where the retained yeast population is large - as intended here - the effect of yeast deterioration at excessive final ethanol concentration *does not become apparent at all rapidly.* The half-life for yeast inactivation may be one or two *days.* It may be possible to find the maximum tolerable level more quickly using lower yeast concentrations, in a *homogeneous* chemostat system; having determined that level, the intensified system should always be operated below that limit. For efficiency, it is better to limit the final ethanol level by limiting the FS input, rather than by operating at unnecessarily high dilution rates, which will stabilize operation but at the cost of converson efficiency.

3-REACTOR SYSTEM FOR IMMOBILIZED YEAST





A three-stage continuous reactor system
(a) for immobilized yeast

We shall now develop in outline a system which uses three of the above (or similar) reactors in series and running continuously. The series feature allows us to divide the total substrate feed, and hence the total feed of inhibitory molasses salts. The three stages provide an element of plug flow in the overall system. On the other hand this same plug flow characteristic will mean that the third reactor operates close to the final conditions where inhibition is maximal; to overcome this effect where an immobilized yeast catalyst is used, the three reactors must be switched at regular intervals, so that the catalyst has optimum conditions for recovery.

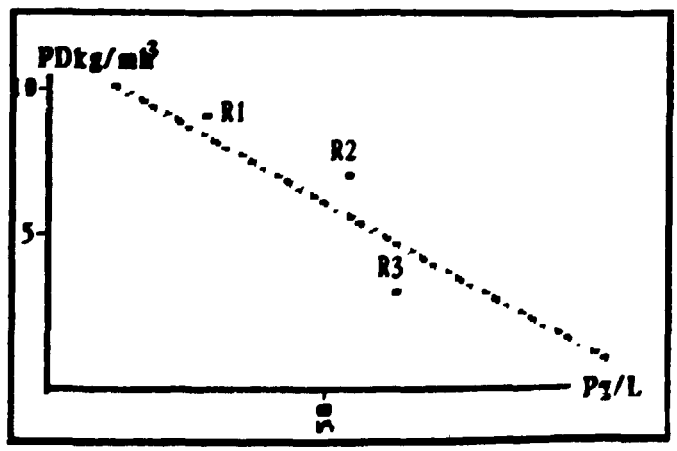
This in turn means that the three reactors must be identical. The pipework for each reactor will be as shown in the following figure, which also shows flow rates calculated as below; the arrangements of valves required for the switching operation is shown as a subsidiary diagram.

The flow quantities shown in the figure are calculated for illustrative purposes on the following basis:

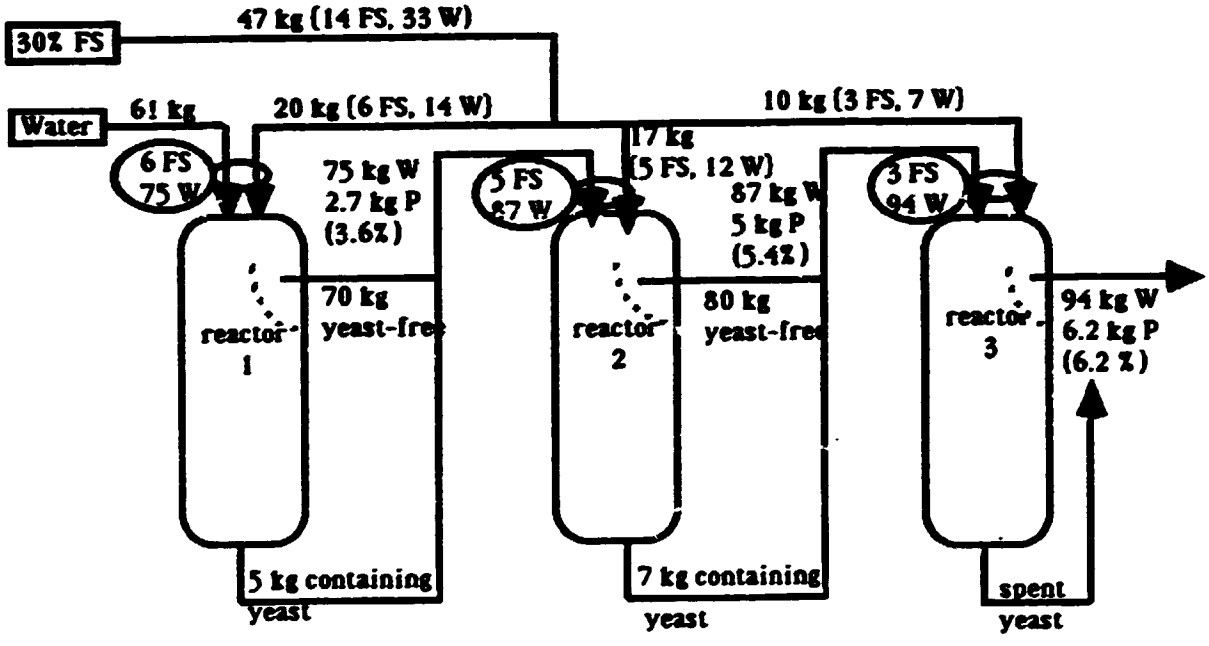
individual reactor volumes 100 L; total working volume 300 L.
assumed flow rate 0.1 h^{-1}, 30 L/h.
flow quantities shown per 100 L throughput, i.e. for 3.3 hours.
feedstock is taken as molasses with 30%FS
yield of ethanol is assumed 0.45 g/g
feedstock diluted to give overall input *ca* 140 g/L FS
substrate feeds to 3 reactors divided in ratio 6:5:3
the effect of ethanol on output weight/volume ratio is neglected.

The loadings of each reactor in terms of molasses salts level and the assumed productivities over the 3.3-hour period are then calculable as shown in the figure, giving an overall productivity of 6.7 kg/m^3h. If the overall flow rate is increased the required individual productivities and the overall productivity are increased in proportion.

Now if we assume the same amount of catalyst in each reactor the specific rates of ethanol production for the three lots of yeast are in proportion to the required productivities, and these can be plotted against the ethanol level thus:



3-REACTOR SYSTEM FOR AUTO-IMMOBILIZED YEAST



Basic Flow Diagram for 3-reactor system using autoimmobilizing yeast. Transfer of yeast requires metered pumps; other transfers by simple overflow.

The figure also shows a broken line which is a plausible indication of the expected effect of P on PD, from which we see that, if anything, the performance of reactor 3 has been under-estimated; this is an appropriate "safety margin" in our design.

(b) with an autoimmobilizing yeast

An essentially similar process arrangement can be proposed for use with an auto-immobilizing - i.e. permanently flocculated - yeast, but in this case the arrangement is somewhat simpler since a proportion of the yeast can quite feasibly be transferred from one reactor to the other, instead of having to switch reactors. The requirement is to divide the flows from reactor 1 to reactor 2, and reactor 2 to reactor 3, into two portions. One flow, usefully taken through a metering pump, is set to a low fraction - say 5-10% - of the total flow rate, and is arranged to transfer both fermentation medium and yeast; this is done by drawing this flow from the mixed volume in the reactor. The remainder is taken free from yeast through the screened exit from the reactor.

It is necessary to arrange some removal of totally spent yeast from the third reactor. In this arrangement the inactivation of yeast in the third reactor becomes automatically balanced by the transfer of fresh yeast from the second, and so on.

(c) two-reactor systems

It should be added that either of these systems can be approached through similar arrangements using only two reactors; in actual trials, it may already be found sufficient for the immediate purposes to adopt such an arrangement, which will of course be automatically available if the reactors are constructed and commissioned one at a time !

A note on auto-immobilizing *versus* immobilized yeast.

Autoimmobilizing yeast provides catalyst particles with a very high yeast density (100% yeast limited only by diffusion effects), which are produced directly from the process raw material (molasses FS) in the reactor itself, which are self-replacing under suitable conditions, and which have the desirable characteristic of being denser than the fermentation medium. Catalysts based on immobilized yeast have a lower yeast density, need to be produced from separate materials (alginate etc.) in a separate reactor, do not self-regenerate but must be replaced by a separate operation if they deteriorate, and tend to lose yeast under yeast growth conditions; their low density causes problems in attempts to operate in the well-mixed mode.

Suggested Immediate Priorities

It is now possible, taking fully into account the existing resources and expertise, to recommend specific tasks and priorities for the NCL team. They are as set out on the following page.

STAGED PRIORITIES FOR THE NCL PROGRAMME

(1) improved, direct, and continued liaison between the microbiology group MB and the process development group PD.

(2a) for PD group, determine whether or not the alginate catalyst can be effectively suspended when actively fermenting, at up to 30% bed volume, by a gas-lift system or a screened impellor. *If not, then to abandon work on alginate immobilization* for this programme.
(2b) for MB group, to make a further assessment of auto--immobilizing yeast strain(s)

(3a) for PD group, build and instal one 100 L reactor and operate first in fed-batch mode, then in continuous but stable mode, to find "conservative" operating limits which may approach those required for the programme target.
(3b) for MB group, yeast selection for surviving CFU from the end of batch cultures run on molasses supplemented with molasses stillage (salts) and ethanol. *note: selection of CFU from autoimmobilizing yeasts can be done from runs with good agitation.* This selection work can also "stand alone" if done in liaison with practical distilleries. It can be continued by selection work using a homogeneous chemostat reactor but the development of a "trophostat" would be more useful and the CFU-survival procedure should have priority.

(4) PD group to build and instal at least one more reactor, to be run first as a parallel fed-batch reactor, then as stage 2 of a 2-stage system; only if proven necessary, build and instal a third reactor for the 3-stage system.

(5) MB and PD groups to collaborate very closely in bringing the programme to a successful conclusion within the allotted period.