BIOREACTORS AND BIOTRANSFORMATIONS

Edited by
G.W. MOODY
and
P.B. BAKER

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INTERNATIONAL CONFERENCE ON BIOREACTORS AND BIOTRANSFORMATIONS GLENEAGLES, SCOTLAND, UK: 9-12 NOVEMBER 1987

Paper A1

OPTIMISATION OF PRODUCT YIELD IN IMMOBILISED PLANT CELL CULTURES

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ABSTRACT

Product yield is critical if plant cell cultures are to be widely used in the production of valuable secondary products in vitro. The immobilisation of freely suspended cells results in advantages in bioreactor configuration and in the physiological state of the cells in some systems. Different matrices can be used in immobilisation including gels of calcium alginate, agarose, agar, polyacrylamide as well as inert support systems such as the hollow fibre reactor and immobilisation in reticulate polyurethane foam. Examples are presented from the production of capsaicin by cultures of Capsicum frutescens of techniques which have resulted in increased product yield. These are: 1. Cell line selection, 2. Immobilisation in foam, 3. Depression of primary metabolism, 4. Alteration of primary metabolism, 5. Induction of activity of rate limiting enzymes, 6. Inhibition of competing reactions, 7. Exploitation of biotransformations, 8. Continuous product removal, 9. Inhibition of product breakdown or further metabolism. Most of these approaches have been attempted and proved successful in Capsicum cultures. Which, if any, of these techniques could prove successful in other species depends on a good understanding of the regulation of the biosynthesis of the desired product.

INTRODUCTION

Between 25% and 30% of prescribed pharmaceuticals are extracted from, or contain ingredients extracted from plant sources [1]. Plants are also used as sources of food colourings and flavourings, and also in the production of agrochemicals. These products supply a market worth billions of dollars per annum and it is not surprising that the possibility of using plant tissue culture systems in order to produce some of these plant secondary products in vitro has received considerable attention over the past decade.

There are several putative advantages in the use of tissue culture over the cultivation of the whole plant in the production of natural products. These include the continuity of, and control over, supply due to the independence of production from environmental factors such as climate and season. The product composition and yield may also be more predictable, and product recovery may be easier in cell culture systems. However, the use of mass cell culture as a means of producing natural products will only find wide acceptance if it offers a cost advantage over conventional systems. In what is a capital intensive system the fundamental problem of this technology is therefore cost of production. This paper discusses product yield which is one critical aspect of production cost.

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Immobilisation

It is now recognised that plant cells in mass culture do not behave like microbial cells and different cultural techniques would be needed to successfully obtain product formation from plant cells in vitro. Plant cells are more susceptible to shear forces than micro- organisms [2] although the importance of this factor is being increasingly questioned (Scragg, pers. comm.). Furthermore, conditions that favour rapid growth tend not to favour product accumulation [3-5]. One solution to these problems is the immobilisation of cells within a solid matrix. Immobilisation binds cells together and protects them from shear and in some instances has beneficial physiological effects which result in increased product yield.

The simplest system of immobilisation is "self-immobilised" cultures which are simply highly aggregated cultures with the cells growing in compact clumps [6]. Manipulation of the culture medium can result in almost 100% of cells growing as aggregates, which in a number of species leads to enhanced secondary metabolite production [7].

Plant cells can also be attached to the surface of inert surfaces with no loss of biosynthetic ability. Cells of Solanum aviculare have been covalently linked to glutaraldehyde activated beads of polyphenylene oxide [8] and Humulus lupulus cells have been attached to a man-made matrix (nylon or polypropylene) [9]. Bornmann and Zachrisson [10] have shown that protoplasts can also be attached to inert supports such as cytodex micro carriers and maintain viability.

By far the most common method of immobilisation is by entrapment, either within a polymeric gel or within a solid support (for reviews see 11 and 12). The gels which have been employed for plant cell immobilisation include calcium alginate, agarose, agar, polyacrylamide, gelatin and carrageenan, of which the first two have generally been found to retain the greatest degree of viability and respiratory and biochemical activity of the cells [13-15]. Because of the mild conditions employed alginate has received most attention, however, high concentrations of Ca^{2+} are required for polymerisation and low phosphate must also be employed. Furthermore, the long term stability of the matrix is poor as cell division leads to break up of the alginate beads.

Techniques for immobilisation which eliminate the need for a gel matrix and depend on an inert mechanical support for the growing culture have recently been developed. The first of these systems which is similar to that employed in some animal cell cultures uses a hollow-fibre reactor in which the cells are trapped in a chamber which is transversed by a large number of semi-permeable membrane tubes through which the medium is pumped [16,17].

The approach used in this laboratory for immobilisation of plant cells in a supporting matrix involves passive entrapment within reticulate polyurethane foam [18]. Blocks of sterilised foam 1 cm^3 are added to suspension cultures and as the cells grow they invade the foam, divide and become entrapped within the pores of the matrix. The advantages of the technique are that the matrix is stable, non-phytotoxic, autoclavable and cheap, and as it is 97% (v/v) void it presents a negligible barrier to permeability. Scale-up of this culture

Species	Matrix	Product of interest	Primary location	Reference
Amaranthus tricolor	Ch	oxalate	M	Knorr & Teutonico (1986)
Asclepias syriaca	Ch	proteases	M	Knorr et al. (1985)
Beta vulgaris	My	betacyanin	C	Rhodes et al. (1985)
Capsicum frutescens	Pf	capsaicin	M	Lindsey & Yeoman (1984)
Catharanthus roseus	Ag	cathenamine	C	Felix et al. (1981)
Coffea arabica	Al	methylxanthines	-	Haldimann & Brodelius (1986)
Daucus carota	Al	phenolics	M	Hamilton et al. (1984)
Digitalis lanata	Al	β-methyldigoxin	M	Alfermann et al. (1983)
Glycine max	Hf	phenolics	-	Schuler (1981)
Glycyrrhiza echinata	Al	echinatin	M	Ayabe et al. (1986)
Hyoscyamus muticus	Pf	atropine	C	Authors (unpub. observ.)
Lavandula vera	Al	pigments	M	Nakajima et al. (1985)
Mentha sp.	Pa	neomenthol	M	Galun et al. (1983)
Morinda citrifolia	Al	anthraquinones	C	Brodelius et al. (1979)
Mucuna pruriens	Al	L-DOPA	M	Wichers et al. (1983)
Nicotiana sylvestris	Pa	monoterpene alcohols	M	Galun et al. (1985)
Nicotiana sp.	Al	cinnamoyl putrescines	C	Berlin (1985)
Papaver somniferum	Al	codein	M	Furuya et al. (1984)
Salvia miltiorrhiza	Al	diterpines	M/C	Miyasaka et al. (1986)
Solanum aviculare	Sb	steroid glyco-alkaloids	M	Jirku et al. (1981)

Table 1. The production of phytochemicals by immobilised plant cell systems. Al (alginate); Ag (agarose); Pa (polyacrylamide); Ch (chitosan); Ny (nylon); Pf (polyurethane foam); Hf (hollow fibre); Sb (surface binding).

C = cells M = medium

system is also possible and bioreactors have been developed which are suitable for foam immobilised cells [19].

Immobilised cell systems in the production of plant secondary products

Immobilised plant cell cultures have a number of advantages over freely suspended cells in the practical field of bioreactor configuration [6,19] and in the physiological state of the cells during the culture period [12]. The high degree of cell aggregation which arises in immobilised systems, results in greater cell-cell contact and possibly communication which act to reduce the rate of cell division and permit the establishment of gradients of nutrients and growth substances which are important in the regulation of secondary metabolic activity. The use of fixed bed reactors which is facilitated by the use of immobilised cells [19] also lends practical advantages to the employment of immobilised cells. Cultural conditions can be changed at will (e.g. from full growth, to nutrient limited, media) in order to shift the balance from conditions favouring biomass production to those favouring secondary product accumulation. The use of circulating medium in a fixed bed reactor also allows the re-use of biomass [12] as well as making possible continuous product removal which in itself leads to increased yields in some systems (see below).

Constraints to the employment of immobilised cultures

The major constraint and limitation to the use of immobilised cultures in the production of secondary metabolites is the requirement for the product to be released into the medium. If the product is retained within the cells the advantages of the fixed bed reactor with circulating medium and continuous product removal no longer apply. However, the limitation to the use of immobilisation technology may prove to be less of a problem than at first anticipated, as Hall *et al* (1987) have shown that the primary location of the product in the majority of reported examples of immobilised cultures is in the medium (Table 1). Furthermore, in several instances the immobilisation of the cells results in a preferential partitioning of the product into the culture medium (Brodelius, pers. comm.).

Permeabilising agents which can cause product release with limited loss of viability have also been considered as part of a culture process [20]. The use of these agents is, however, outwith the scope of this article but the reader is referred to the review of Parr *et al*. [20].

Increased product yield from immobilised cultures

Given the constraints of the need for product release, several techniques have been developed which can lead to increased yields of desired secondary products in cultures. All of the following examples are taken from the experience in this laboratory in the production of capsaicin in cultures of *Capsicum frutescens* but the authors believe that with a good understanding of biosynthetic pathways, these general principles can be applied to any desired plant secondary product. The selection of high yielding cell lines and their stability over prolonged periods of culture is obviously of great importance in successful process development but discussion of these subjects is, however, outwith the scope of this article.

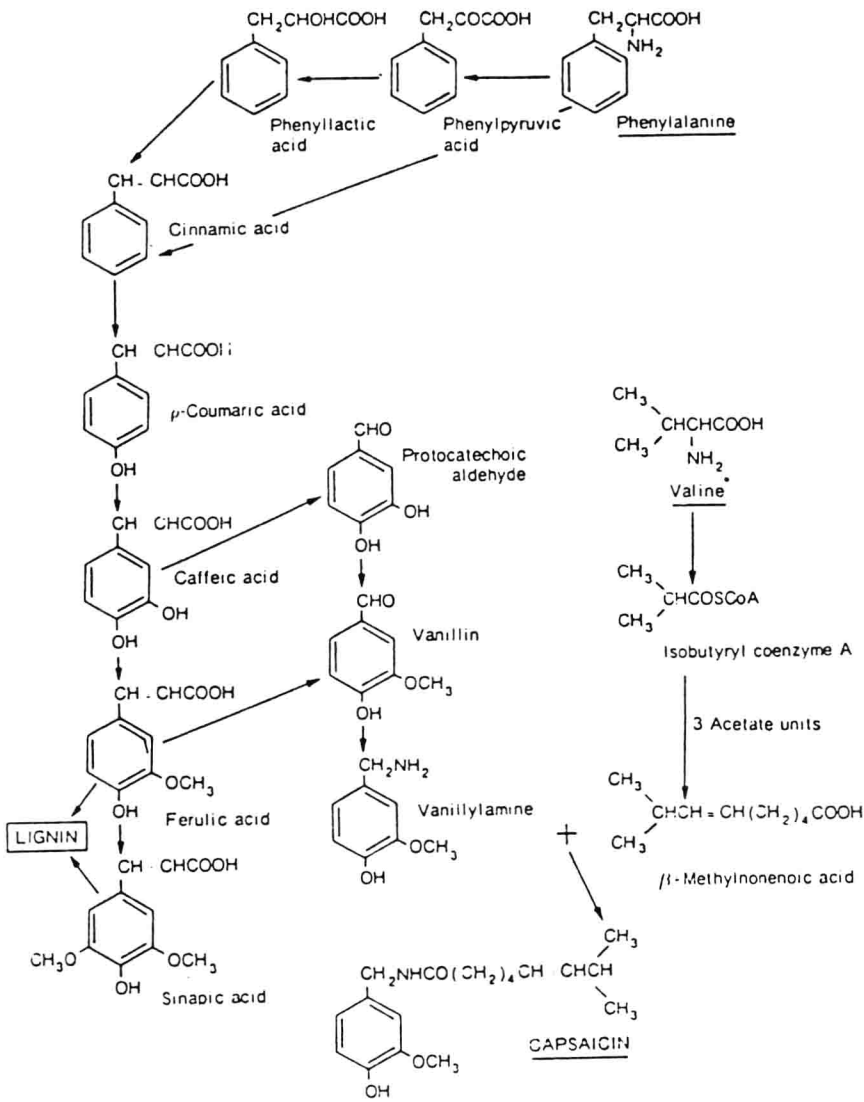

Figure 1. Proposed biosynthetic pathway for capsaicin from phenylalanine and valine. (After Yeoman et al. 1980)

Reduction in, or alteration to primary metabolism

From Fig. 1 it can be seen that one branch of the capsaicin biosynthetic pathway is derived from phenylalanine and one from valine. As these compounds are amino acids, they are largely incorporated into proteins when cultured under conditions which favour rapid cell growth [5,22]. If, however, cultures are grown under conditions of nutrient limitation which reduce growth rate, larger pools of these precursors are available for secondary metabolite production. Conditions which limit growth (i.e. absence of nitrate, phosphate and growth regulators) can therefore be used in order to increase product accumulation. Table 2 shows that incorporation of radioactivity from ^{14}C phenylalanine is directly proportional to the initial nitrate concentration in the media. Thus at high nitrate levels incorporation into protein is high and incorporation into capsaicin is low and vice versa. The preferential production of the desired secondary product can therefore be achieved by using conditions of nutrient limitation [23]. Although not a universal observation, the inverse relationship between cell growth and product formation has been observed in several systems [12].

Table 2. The effect of initial nitrate concentration on the incorporation of [^{14}C]Phe into soluble protein and capsaicin in cultures of immobilized pepper cells after 9 d. Cultures (20 ml) were supplied with 74 kBq of L-[U-^{14}C]Phe for 24 h. Results represent the mean of three replicates ± SE.

Initial NO_3 (mM)	Radioactivity in soluble protein		Radioactivity in capsaicin	
	mean (Bq g^{-1} DW)	% Full	mean (Bq g^{-1} DW)	% Full
0	4481 ± 695	34	484 ± 68	272
5	5892 ± 655	45	375 ± 83	210
10	8646 ± 374	66	166 ± 45	93
25	13044 ±2784	100	178 ± 33	100
50	23870 ±1814	183	111 ± 19	62

After Lindsey [23]

As the supply of phenylalanine has been shown to limit capsaicin accumulation in immobilised cultures, other techniques can be developed to increase the supply of this key precursor. Cell lines have spontaneously arisen in cultures of different species which produce greatly elevated levels of certain amino acids including phenylalanine. These over-producers can be conveniently selected by growing treated or non-treated calli on media containing synthetic analogues of phenylalanine. As the analogues are toxic, cultures which survive may have elevated levels of endogenous phenylalanine. The selection of phenylalanine over-producers may therefore result in cell lines with increased capsaicin accumulation [24].

Increased activity of an enzymic step between primary and secondary metabolism

Phenylalanine ammonia-lyase (PAL) converts phenylalanine to cinnamic acid in the first step of the phenylpropanoid branch of the capsaicin

biosynthetic pathway. This enzyme is considered to be a key switchpoint between primary metabolism and the phenolic pathways of secondary metabolism, and several examples can be cited of increased enzymic activity leading to increased product synthesis [25]. PAL activity can be induced by many environmental factors, including low temperature, illumination with UV light, dilution of the biomass and the addition of fungal elicitors [25]. Of these treatments, the use of fungal elicitors has found favour with an increasing number of workers.

The use of elicitors is especially relevant if the product of interest is a phytoalexin such as phaseolin, but other compounds which are not thought to be phytoalexins can also show increased yields in response to elicitor treatment.

In the example of Capsicum cultures, it has been shown that spores of Monilinia fructicola can increase both capsaicin accumulation and incorporation of radioactive precursors into the product (Lindsey and Holden, unpublished observations). Present studies are under way to determine if this effect is mediated by increasing PAL activity.

Inhibition of competing secondary reactions

It has been shown that a high proportion (up to 63%) of supplied phenylalanine is not incorporated into capsaicin but is instead utilised in cell wall synthesis, either as esterified hydroxy-cinnamic derivatives or into lignin [22]. It may prove possible to divert intermediates into capsaicin synthesis by a number of treatments.

If excess product of a competing pathway is supplied to cultures, the equilibrium of the reaction can be shifted in favour of substrate accumulation. Sinapic acid which is derived from ferulic acid (an intermediate in the capsaicin pathway [Fig. 1]) can be fed to Capsicum cultures and results in increased capsaicin accumulation [19]. Feedback inhibition probably results in reduced demand for ferulic acid which then becomes available for capsaicin biosynthesis.

Exploitation of biotransformations

One or two step reactions may result in the synthesis of a high-value compound from a low-value precursor. Simple biotransformations such as the glycosylation of digitoxin to purpurea glycoside A and the hydroxylation of β-methyldigitoxin to β-methyldigoxin can be performed over long culture periods in immobilised cells [26]. The L-tyrosine to L-Dopa biotransformation can also be successfully achieved with cultured immobilised cells of Mucuna puriens [27]. This use of immobilised cultures is obviously only economically viable if the product is substantially more expensive than the supplied substrate and if the product is released into the culture medium. In the capsaicin pathway the ferulic acid to vanillin biotransformation (Fig. 1) meets these criteria. Vanillin is a high cost flavouring used extensively in the food and drinks industries and therefore has the potential to be exploited in culture systems. In our laboratory cell lines have been established which preferentially release this product into the medium and the full biosynthetic potential of these cultures is presently under investigation.

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