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PRODUCTION OF L-ASPARAGINASE FROM SOIL ACTINOMYCETES AND ITS ANTILEUKAEMIC POTENTIAL

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Abstract

L-Asparaginase has been part of the treatment of acute lymphoblastic leukaemia for decades, but the bacterial enzymes in clinical use cause hypersensitivity, antibody formation and other toxicities, which keeps interest alive in new microbial sources. This narrative review summarises published work on soil actinomycetes as producers of L-asparaginase and on the antileukaemic potential of their enzymes. It covers isolation and plate screening, fermentation conditions and statistical optimisation, purification and enzyme properties, and cell-line and mechanistic evidence for antileukaemic activity. No new experiments or data are reported. Most evidence is preclinical, glutaminase activity and immunogenicity are rarely assessed, and few actinomycete enzymes have been compared directly with licensed products.

1. INTRODUCTION

The story of L-asparaginase as an anticancer enzyme starts with an observation, not a design. Kidd noticed that guinea pig serum could make transplanted lymphomas in mice regress [1], and Broome later traced this effect to the enzyme L-asparaginase in the serum [2]. Within a few years, the enzyme from Escherichia coli was shown to have the same effect in animal models [3]. The reasoning is simple. Some leukaemic lymphoblasts cannot make enough of the amino acid asparagine for themselves, so they depend on the supply in blood plasma, and an enzyme that hydrolyses asparagine to aspartic

acid and ammonia starves them [4, 5]. Normal cells, which can synthesise their own asparagine, are spared to a greater extent. Early clinical trials in the 1960s and 1970s then confirmed activity in children with leukaemia, and the enzyme moved into combination protocols alongside vincristine, corticosteroids and anthracyclines. The simplicity of the idea is part of its appeal: a single protein that removes one nutrient from the blood [4, 6]. Few areas of cancer therapy owe so much to a laboratory curiosity.

That logic has held up well in the clinic. Asparaginase preparations from E. coli and Erwinia chrysanthemi, and a polyethylene glycol conjugate of the E. coli enzyme, are part of standard multi-agent regimens for acute lymphoblastic leukaemia (ALL), especially in children, where they contribute to the high cure rates now achieved [6, 7, 8]. The difficulty is that these enzymes are foreign proteins. Hypersensitivity, neutralising antibodies, pancreatitis, thrombosis, liver dysfunction and other adverse effects limit their use in some patients, and switching between preparations is a common clinical response [7, 8, 9]. Pegylated and Erwinia preparations were introduced precisely to deal with allergic reactions and antibody-mediated inactivation, and trials comparing native enzymes showed differences in the depth and duration of asparagine depletion between products. The need for an alternative source has therefore been framed in clinical, not only commercial, terms [9, 10]. Long-term data have also shown that completion of the planned asparaginase course is associated with better outcomes, so tolerance matters [6, 8]. Expanding the supply of tolerable enzyme is therefore a practical aim.

Because of these limits, researchers have looked for enzymes from other microbial sources with different immunological or kinetic properties. Soil actinomycetes, and Streptomyces in particular, are attractive because they are abundant, easy to culture and well known for secondary metabolites and extracellular enzymes [11, 12, 13]. This article reviews the published literature on L-asparaginase from soil actinomycetes and on its antileukaemic potential. It is a narrative review reporting no new data. The sections cover isolation, production, enzyme properties and anticancer evidence, with the main findings compiled in table 1 and grouped in fig. 1. The topic also has a practical side for laboratories in countries where imported enzyme is costly, since local production from cheap soil isolates is sometimes presented as a route to affordable supply [14, 15]. The review is organised around the steps a research group would follow from soil sample to leukaemia cell line [14, 15].

2. MICROBIAL SOURCES AND SCREENING

Actinomycetes are Gram-positive filamentous bacteria with high DNA guanine and cytosine content, and they are among the commonest organisms in soil, where they take part in the breakdown of organic matter and produce a remarkable array of antibiotics and enzymes [11, 16, 17]. Genomic surveys of the phylum show large genomes with many biosynthetic gene clusters, which explains why screening programmes have so often turned to them [12, 16]. Most of the L-asparaginase literature on actinomycetes concerns the genus Streptomyces, though other genera are occasionally reported from rhizosphere and marine habitats [12, 18]. Streptomyces species alone account for a large share of known microbial antibiotics, and this reputation helped to prompt screening of the group for enzymes of medical interest, such as asparaginase, as well [11, 13]. Soil actinomycetes are therefore a reasonable place to look, though abundance alone does not guarantee a suitable enzyme [12, 17]. Whether an actinomycete enzyme can match the properties of the established products remains the open question.

Table 1: Published findings on microbial L-asparaginase relevant to soil actinomycetes

Topic	Reported finding	Ref.
Origin of the concept	Guinea pig serum asparaginase was linked to regression of transplanted lymphomas	[1, 2]
Clinical use in ALL	Enzyme preparations form part of standard regimens, especially in children	[6, 7, 8]

Topic	Reported finding	Ref.
Adverse effects	Hypersensitivity, antibodies, pancreatitis and thrombosis limit use in some patients	[7, 8, 9]
Plate screening Phenol red or bromothymol blue	Colour change from ammonia release allows rapid screening of isolates	[19, 20]
Statistical optimisation	Plackett-Burman and response surface designs raise yields in flask culture	[21, 22]
Activity assay	Nessler method estimates ammonia release; protein by Lowry or Bradford	[23, 24, 25]
Enzyme structure	E. coli enzyme is a tetramer with a conserved active site	[26]
Kinetic affinity	Therapeutic enzymes need micromolar affinity for asparagine	[9, 26]
Glutaminase side activity	Linked to some toxicity; glutaminase-free enzymes sought	[9, 27]
Cell-line studies	Purified Streptomyces enzymes reduce viability of leukaemic lines in culture	[14, 15, 18]
MTT viability assay	Common endpoint for cytotoxicity, sensitive to culture conditions	[28]

Isolation usually begins with soil collected from varied sites, air-dried to reduce Gram-negative contaminants, diluted serially and plated on a selective medium such as starch casein agar or glycerol asparagine agar, often with antifungal agents added to suppress moulds [17, 29]. Colonies with the chalky, aerial-mycelium appearance typical of actinomycetes are purified by streaking. Tentative genus assignment relies on morphology, spore chain arrangement and colour series, following the International Streptomyces Project criteria [29, 30]. Molecular identification by 16S ribosomal DNA sequencing is now regarded as the minimum standard for a robust report [31]. The incubation period for colonies on selective agar is several days, which is slow compared with bacteria such as E. coli and slows the whole workflow. Soil from agricultural fields, forests and rhizosphere sites has all been used, and there is little evidence that any particular habitat gives a superior enzyme [12, 17]. Careful records of the soil source, medium and incubation conditions make later work easier to reproduce [17, 29].

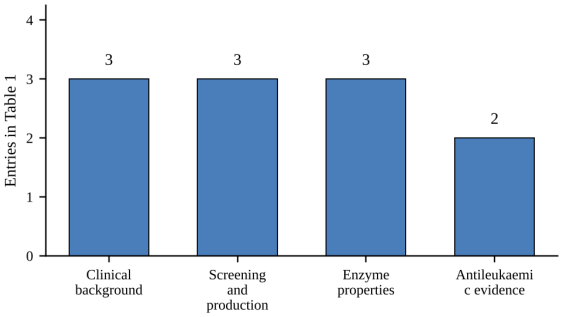


Fig. 1: Number of entries in table 1 by topic group

The workhorse for primary screening is the rapid plate assay. Gulati and colleagues described a medium containing L-asparagine and the pH indicator phenol red, in which hydrolysis of asparagine releases ammonia, raises the pH and turns the colony surroundings from yellow to pink [19]. A later modification used bromothymol blue as the indicator and was reported to be more sensitive [20]. Plate assays are quick and cheap, and they allow hundreds of isolates to be examined. They are also qualitative. Zone size does not map neatly onto enzyme yield, and a pH shift can also arise from other ammonia-producing metabolism, so positive isolates must be confirmed in broth [19, 20, 32]. Some groups skip the plate assay and go directly to broth culture of selected isolates, which saves time but gives up the advantage of wide screening. In either case the key control is an uninoculated plate or flask, since the indicator can change colour by itself if the medium is not buffered correctly [19, 20]. The practical lesson is to treat the plate result as a pointer rather than a measurement [19, 32].

3. PRODUCTION AND OPTIMISATION

Enzyme production by submerged fermentation is influenced by medium composition, pH, temperature, inoculum, aeration and incubation time. Published studies on Streptomyces report that L-asparagine itself, or another nitrogen source in the medium, acts as an inducer, and that production tends to fall when readily metabolised nitrogen such as ammonium or glutamine is plentiful [18, 32]. Carbon

sources such as glucose, starch and glycerol have varied effects depending on strain, so what works for one isolate cannot be assumed to work for the next. Neutral to slightly alkaline starting pH and mesophilic temperatures are most often reported as favourable [18, 27]. Incubation time matters because extracellular activity usually peaks late in growth and may decline as proteases accumulate in the broth. Reports commonly collect samples at intervals of 24 hours or more to find this peak, which is a sensible precaution for filamentous organisms with slow growth [12, 32]. Reports that vary several factors together are more convincing than those that vary one at a time [21, 22].

Because several variables interact, one-factor-at-a-time experiments are inefficient and can miss synergies. Statistical designs, notably the Plackett-Burman screening design followed by response surface methodology on the few influential variables, have been widely used for microbial enzyme optimisation, and they have been applied to asparaginase production [21, 22]. The usual outcome is a multi-fold rise in activity compared with the starting medium. Such gains are real, but they are specific to the strain and flasks used, and they do not predict performance in a stirred bioreactor where oxygen transfer and shear differ [21, 22]. Software used for these designs reports an equation relating activity to the coded variables, and an analysis of variance to judge the fit. A model that is not tested by a confirmatory run at the predicted optimum is only half finished, yet confirmation is not always described [21, 22]. In any given paper the yield gain should be read alongside the starting medium, which is often a weak baseline [21, 22].

Measurement of activity most often relies on the Nessler method, in which ammonia released from asparagine is estimated colorimetrically, with one international unit defined as the amount of enzyme releasing one micromole of ammonia per minute under stated conditions [23, 32]. Total protein is measured by the Lowry or Bradford methods so that specific activity can be calculated [24, 25]. Reports differ in their buffers, substrate concentrations and temperatures, so activity values from different papers can rarely be compared directly. Interference from ammonia already present in culture broth is a further source of error that careful blanks must control [23, 24]. Many reports also express activity per millilitre of broth rather than per gram of cells, which obscures differences in growth between media and makes comparisons between strains less reliable than they look [23, 32]. Reporting the assay conditions in full is the simplest remedy for this problem [23, 25].

Scale-up is rarely addressed in actinomycete papers. Filamentous growth makes the broth viscous, pellet formation changes oxygen transfer, and extracellular enzyme release depends on the growth phase [12, 13]. A process that gives good titres in shake flasks may behave differently in a fermenter, and without data on yield per litre and per gram of biomass the commercial potential of a strain is hard to judge [15, 21]. Commercial fermentations of recombinant E. coli, by contrast, reach high cell density with well-defined controls, which sets a demanding benchmark for natural isolates [14, 15]. Stated yields should accordingly be treated as preliminary.

4. PURIFICATION AND ENZYME PROPERTIES

Purification of extracellular enzyme from actinomycete culture typically follows a familiar sequence. The cell-free supernatant is concentrated by ammonium sulphate precipitation, dialysed, and passed through ion-exchange and gel filtration columns, with fractions followed by activity and protein assays [4, 14, 27]. Purity is checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis, which also gives an estimate of subunit size [33]. Reported purification folds and recoveries vary widely among studies and should be read with care, as they depend on the starting activity and the stage at which fractions were pooled [14, 15]. Heat treatment or pH adjustment sometimes precedes chromatography as an inexpensive way to remove contaminating proteins, provided the enzyme is stable enough to survive it. Where it is not, losses can exceed the benefit and recovery falls sharply [4, 14]. Reports usually give a table of each step with

total activity and specific activity, which allows the reader to judge losses [4, 27].

The properties reported for microbial asparaginases give a frame of reference. The E. coli enzyme is a tetramer of identical subunits of roughly 34 kDa, each with an active site that binds asparagine through conserved residues [26]. The Erwinia enzyme is similar in architecture, with different surface properties and a shorter half-life in plasma [7, 34]. Studies on actinomycete enzymes usually report a subunit size in the same general range, an optimum pH between neutral and alkaline, optimum temperatures around those of mesophiles, and moderate stability, though purified enzymes are not always shown to be tetrameric [14, 15, 18]. Comparisons with E. coli and Erwinia enzymes show that small differences in surface charge and loop structure can alter stability and immunological behaviour considerably, which is part of the reason a new source is thought to be worth examining [26, 34]. In short, a purified protein of the right size is a necessary starting point, not proof of therapeutic suitability [15, 26]. Thermal stability is a further point of difference, since some actinomycete enzymes are reported to tolerate higher temperatures than the clinical products [15, 18].

Kinetic constants determine whether an enzyme could work in blood. Therapeutic enzymes have a Michaelis constant for asparagine in the micromolar range, so that plasma asparagine can be depleted to a very low level [9, 26]. Some actinomycete enzymes have been reported with values in the millimolar range, which would make them poor candidates unless engineered [14, 15, 27]. Substrate specificity is also important. Many asparaginases show side activity against glutamine, and glutaminase activity is blamed for part of the toxicity of the clinical enzymes, including effects on the liver, brain and pancreas, which is why glutaminase-free preparations are sought [9, 27]. Metal ions and inhibitors can modulate activity, and reports on actinomycete enzymes list the effects of cations and chelators with inconsistent results, perhaps because buffer composition and enzyme purity differ so much among laboratories [14, 15, 18]. Such values must be measured, not assumed from a related organism [9, 27].

Table 1 summarises representative findings on sources, assays, enzyme properties and therapeutic use drawn from the references discussed above, and fig. 1 shows the distribution of entries by topic. The table makes plain that background knowledge on clinical asparaginases is rich, whereas the data on actinomycete enzymes are scattered and rarely directly comparable with licensed products [4, 7, 14, 15]. For the purposes of this article, the table is a map of the literature rather than a ranking of enzymes. Gaps in the table are as informative as the entries.

5. ANTILEUKAEMIC POTENTIAL

The mechanism of action is well understood. Lymphoblasts from many patients with ALL have low expression of asparagine synthetase and cannot meet their needs by internal synthesis, so depletion of plasma asparagine leads to inhibition of protein synthesis and cell death by apoptosis [4, 5, 8]. Clinical practice uses the depth and duration of asparagine depletion as a pharmacodynamic marker, and the monitoring of serum asparaginase activity is used by some groups to detect silent inactivation by antibodies [8, 9]. These ideas set the standard against which any new enzyme would be judged. Resistance in the clinic is partly pharmacological and partly biological. Antibodies clear the enzyme quickly, while leukaemic cells may increase their own asparagine synthesis in response to depletion. Both mechanisms apply equally to any new enzyme, whatever its origin [4, 7, 8]. Any claim of potential should be measured against these benchmarks [5, 9]. Dose scheduling in the clinic is built on this logic.

Preclinical work on actinomycete enzymes usually uses human leukaemia cell lines, with cell viability measured by the MTT assay, in which metabolically active cells reduce a tetrazolium dye to a coloured formazan [28]. Several studies have reported that purified Streptomyces enzymes reduce viability of leukaemic lines in a concentration-dependent way, and some compare the effect with a commercial E. coli enzyme [14, 15, 18]. These findings are encouraging as a first step. They show that the enzyme is active against

cells in culture, but not that it would do so in a patient [5, 15]. Primary patient samples tested ex vivo are a more informative model than established lines, because they retain the heterogeneity of the disease, although such experiments are scarcely described for enzymes from soil actinomycetes [5, 8]. A cautious reading treats such data as supporting evidence, not as proof of efficacy [15, 28].

Several caveats apply. Culture media contain asparagine and glutamine at concentrations that differ from plasma, so cell-line sensitivity depends on the medium used [5]. Cell lines differ in asparagine synthetase expression, and resistance in the clinic can arise from upregulation of this enzyme [4, 8]. Comparisons that lack a commercial enzyme control, matching the same units of activity, are difficult to interpret. Studies seldom report endotoxin levels, which can themselves be toxic to cells and confound viability data [15]. An enzyme with good activity in a flask of culture medium may therefore behave quite differently once it meets plasma proteins and clearance mechanisms [9]. Appropriate controls make the result easier to trust [5].

Immunogenicity is the central reason for seeking new enzymes and the one that is least studied. Hypersensitivity reactions and neutralising antibodies arise because the clinical enzymes are bacterial proteins, and switching to the Erwinia enzyme when E. coli product fails works partly because the two share limited antigenic overlap [7, 10]. A new actinomycete enzyme might avoid cross-reactivity with both, but that has to be shown with serum from treated patients, and no such work has been reported for soil actinomycete enzymes [7, 10]. Such serological work needs patient samples and ethical approval, which is one reason it is seldom attempted in basic research settings [7, 10].

6. LIMITATIONS AND FUTURE DIRECTIONS

The evidence base for soil actinomycete asparaginase has clear limits. Most studies are single-laboratory descriptions of one isolate, with in vitro cell data and no animal models [14, 15, 18]. Few report glutaminase activity, thermal stability in serum, kinetic constants at physiological substrate concentrations or immunogenicity, which are exactly the properties that distinguish a promising therapeutic enzyme from an industrial one [9, 26, 27]. Direct, matched comparison with licensed products is rare, and unit definitions differ between papers [7, 23]. Many of the same papers also quote high specific activities that were measured in crude preparations, a figure that is tempting to compare with purified clinical products but should not be [14, 15]. These limits explain why few of these enzymes have been taken beyond laboratory study [7, 15].

A more rigorous programme would start with sequence data, so that the enzyme can be classified and compared with known type II enzymes [26, 31]. It would measure kinetics at low substrate levels, test glutaminase activity, check stability in human serum, and assess immunological cross-reactivity before any claim of antileukaemic promise is made. Protein engineering and PEGylation may then be used to extend half-life [9, 34]. Clinical translation, if it comes, will depend on this chain of evidence, not on yield alone [8, 15]. Collaboration between microbiology groups and clinical pharmacology laboratories would speed this process, since the latter hold the assays for depletion and antibody monitoring [9, 10]. Honest reporting of negative results would help others avoid repeating dead ends [15, 27].

7. CONCLUSION

Soil actinomycetes are easy to isolate and clearly capable of producing extracellular L-asparaginase, and screening, medium optimisation and purification are well documented [14, 18, 19, 21]. Cell-culture work suggests that some of these enzymes can reduce the viability of leukaemic lines, which fits the established mechanism of asparagine depletion [4, 5, 15]. These results justify further study. They do not yet justify any claim that an actinomycete enzyme is a therapeutic candidate. The field is still at the stage of screening and characterisation, and progress is limited by the lack of shared methods.

The properties that matter clinically are the ones least often reported: affinity for asparagine at plasma concentrations, glutaminase activity, half-life in serum and immunogenicity [9, 10, 26, 27]. Studies that address them, with matched comparison against licensed enzymes and with proper controls for endotoxin and culture medium, would carry the field forward [7]. Until then, the enzyme from soil actinomycetes is better described as an interesting research lead. Reviewers and readers should expect these questions to be answered before a clinical case is made.

8. ABBREVIATIONS

ALL-Acute lymphoblastic leukaemia, DNA-Deoxyribonucleic acid, MTT-3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PEG-Polyethylene glycol, IU-International unit, kDa-Kilodalton, SDS-PAGE-Sodium dodecyl sulfate polyacrylamide gel electrophoresis, RSM-Response surface methodology

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10. AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

11. CONFLICT OF INTERESTS

Declared none

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