Reflections on Biochemistry

The discovery of β-galactosidase

R. J. Rouwenhorst, J. T. Pronk and J. P. van Dijken

The enzyme β-galactosidase was first mentioned in the literature by Beijerinck exactly a hundred years ago[1]. The Department of Microbiology and Enzymology of the Delft University of Technology keeps the memory of Beijerinck, its first professor, alive by maintaining a 'Beijerinck-room' in the attic of the building. In addition to manuscripts and laboratory notebooks, this room contains some of his chemicals and biological preparations, and it was here that we recently found a 90-year old lactase preparation. Even after storage under suboptimal conditions, the preparation still exhibited measurable enzyme activities.

Lactase

β-Galactosidase (lactase; EC 3.2.1.23) is a well known and extensively studied enzyme which catalyses the hydrolysis of milk sugar (lactose) into the monosaccharides D-galactose and D-glucose. Lactase is produced by a wide variety of organisms including bacteria, yeasts and fungi. Lactases of yeasts and filamentous fungi are of industrial importance in the saccharification of whey permeate, allowing the subsequent alcoholic fermentation with *Saccharomyces cerevisiae*. The enzyme is also applied in the treatment of skimmed milk to allow its consumption in developing countries where the incidence of lactose intolerance is high.

The lactose operon in *Escherichia coli*, first described in 1961 by Jacob and Monod, has had an enormous impact on modern molecular genetics. At present, the *lac*Z gene and *lac*I–Z fusions are widely used as indicators of gene integration and promoter activity. Lactase, encoded by the *lac*Z gene, can easily be detected by using the artificial substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG).

Lactase was among the first hydrolases to be discovered. In the 1880s and 1890s, many enzymes were described. In most cases yeasts (the word enzyme literally means 'in yeast') were used as a source of these proteins (e.g. invertase, maltase and trehalase). The first report that yeast cells may split lactose enzymatically into its hexose constituents was published by Beijerinck in 1889 (Fig.1).

Beijerinck as a microbiologist

Martinus Willem Beijerinck started his scientific career as a teacher of botany in 1873 at the Agricultural School of Wageningen, The Netherlands. After 12 years he became an industrial microbiologist at the Dutch yeast factory in Delft*. In 1895 he returned to the academic world as professor of Bacteriology at the Polytechnical School in Delft, a position he held until his retirement in 1921.

During his scientific career, Beijerinck published over 100 articles dealing with a great variety of subjects in the fields of botany, microbiology and virology. His scientific achievements include fundamental papers on the physiology of luminescent bacteria, the root nodules of Leguminosae and bacterial nitrogen fixation. Beijerinck successfully applied microbiological methods to the study of unicellular green algae, zoochlorellae and lichen gonidia, thereby achieving for the first time pure cultures of these organisms. Beijerinck's work on tobacco mosaic disease may be considered to mark the beginning of modern virus research. He established properties which later appeared characteristic of all viruses: multiplication in dividing tissue cells, transfer of infection by virus-containing fluid, inactivation by heating, and viability after drying or ethanol precipitation. Further topics of research include the discovery of yeast *Schizosaccharomyces octosporus*, studies on the butyl alcohol fermentation, investigations into the microorganisms of milk and other dairy products, and systematic studies on acetic acid bacteria and sulphate reducers. Beijerinck also made extensive investigations on the nutritional requirements of microorganisms and developed new techniques for studies in this field (the auxanographic method). A very important contribution to general microbiology was the development of the enrichment principle. Beijerinck and Winogradsky were the first to apply the idea that culture conditions such as medium composition lead to selective enrichment from natural samples of those microbes that are optimally adapted to these conditions.

β-galactosidase 100 years ago

The fermentation of the milk sugar lactose was a subject which attracted Beijerinck's interest while working at the Dutch yeast factory in Delft. He isolated two yeast species, *Saccharomyces kefyr* and *Saccharomyces tyrocola*, that were able to ferment lactose[2] (*S. kefyr* has been renamed *Kluyveromyces marxianus* var. *marxianus*; *S. tyrocola* has not yet been classified[3]). For the detection of disaccharide-splitting enzymes in microorganisms, Beijerinck developed a very elegant bioassay based on the ability of *Photobacterium phosphorescens* (renamed as *Photobacterium phosphoreum*) to emit light when hexoses are available as a source of carbon. However, the bacterium is unable to use disaccharides directly. When plating a suspension of the photobacteria on gelatine slants supplemented with lactose, Beijerinck

Die Lactase, ein neues Enzym.

Von

M. W. Beyerinck

in

Delft.

Mit 2 Figuren.

Fig. 1. Title page of Beijerinck's paper[1] on the discovery of lactase in the yeasts S. kefyr *and* S. tyrocola.

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*A factory then called 'Nederlandsche Gist-en Spiritusfabriek' and at present known as 'Koninklijke Gist-brocades NV Delft'.

did not observe growth or luminescence. However, when cells of *S.kefyr* or *S. tyrocola* were plated together with bacteria, light emission could be observed around the yeast colonies. From these 'luminescent plates', Beijerinck concluded that lactose fermentation by the yeast is preceded by enzyme-catalysed hydrolysis of the disaccharide and, furthermore, that the responsible enzyme activity (which he named lactase) was secreted by the yeast cells into the environment. In the original paper, there is also a brief description of the procedure by which the lactase preparations were obtained. In essence, an *S. kefyr* culture was filtrated, and then the enzyme activity was precipitated with 85% ethanol. Using this bioassay but with cane sugar instead of milk sugar as a carbon source, Beijerinck demonstrated that these crude lactase preparations also possessed sucrose-inverting activity. He concluded that lactase was capable of hydrolysing both lactose and sucrose[1]. Peculiarly, the lactose-hydrolysing activity of the ethanol precipitate was not mentioned explicitly: only the sucrose-hydrolysing activity of the preparation was described.

In the years following these observations, considerable doubt was expressed in the literature as to the validity of Beijerinck's conclusions. These doubts were caused by the ambiguous results that may be obtained with Beijerinck's bioassay. Using a different (chemical) method for the detection of monosaccharides, Schuurmans Stekhoven could not detect any lactose-hydrolysing activity in culture fluids of *S. kefyr*, nor could he solubilize any such activity from fresh or dried cells by treatment with water at 30°C[4]. This author therefore concluded that the luminescence observed in Beijerinck's experiments with lactose could not be based on the metabolism of monosaccharides by the photobacteria. Instead, he suggested that the photobacterium used glycerol[4], a product of lactose fermentation by the yeast. Moreover, Schuurmans Stekhoven pointed out that the dual activity of Beijerinck's lactase was a misinterpretation. He found that kefyr yeast produces an extracellular enzyme, distinct from lactase, capable of inverting sucrose. A few years later Fischer 'rediscovered' lactase[5]. The enzyme could be solubilized from *S.kefyr* cells by treatment with glass beads or toluene and its activity detected by chemical methods. These observations demonstrated the intracellular localization of lactase in yeasts, a fact now generally accepted. Fischer[6] concluded that Beijerinck's observations had not provided conclusive evidence for the presence of lactase in *S. kefyr*.

The bottle in the attic

Among other preparations from Beijerinck's time, we recently found a small stoppered flask dated 4 December 1899 (see Fig. 2). According to the label, the stoppered flask contained a dried lactase preparation. Indeed, from his hand-written laboratory notebooks it can be concluded that ten years after his discovery of lactase, Beijerinck returned to his studies of lactose utilization by microorganisms. The conditions under which the preparation was stored in Beijerinck's laboratory is not known. However, over the last 30 years it has been kept in the attic of the current laboratory building and exposed to widely fluctuating temperatures (between approximately −10°C and 40°C).

The lactase preparation consists of a dry powder of brownish colour. By using a very sensitive, enzymatic alcohol assay[7], we were able to demonstrate the presence of traces of ethanol in this preparation. This probably reflects the use of ethanol for the precipitation of lactase, a procedure mentioned in Beijerinck's first description of the enzyme. Phase contrast microscopy revealed that the lactase preparation was not cell-free: large numbers of yeast cells could be seen (Fig. 3).

Fig. 2. A stoppered flask containing lactase, found in one of Beijerinck's former cabinets. The label shows the date of preparation: 4 December 1899.

Old enzymes never die

Surprisingly, after 90 years of storage under sub-optimal conditions, the lactase preparation still exhibited measurable hydrolase activities. When assayed with ONPG as a substrate, the preparation showed a low but signifi-

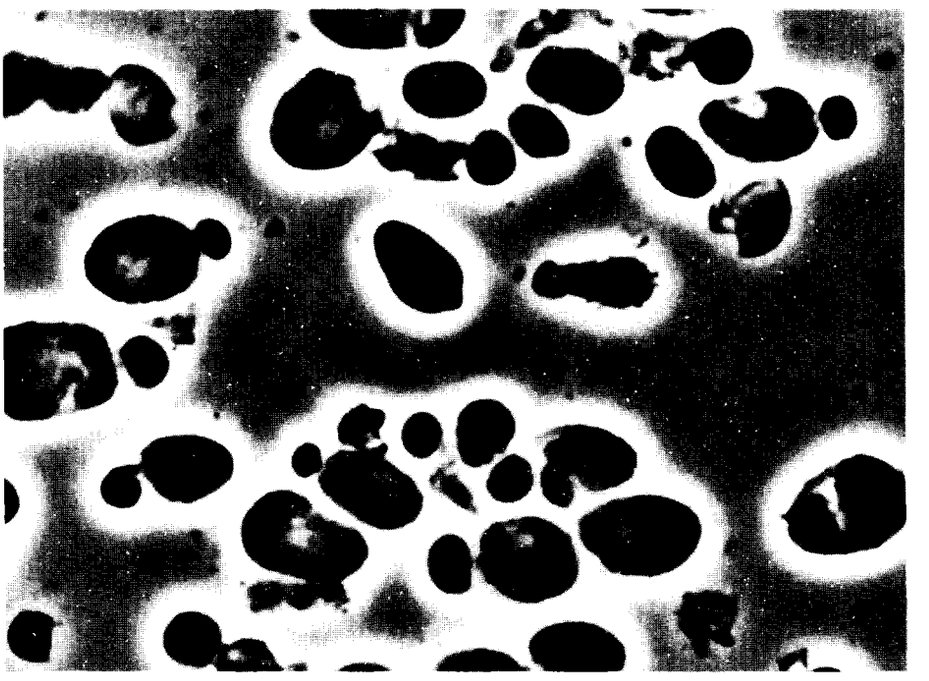

Fig. 3. Phase contrast photomicrograph of yeast cells present in Beijerinck's lactase preparation.

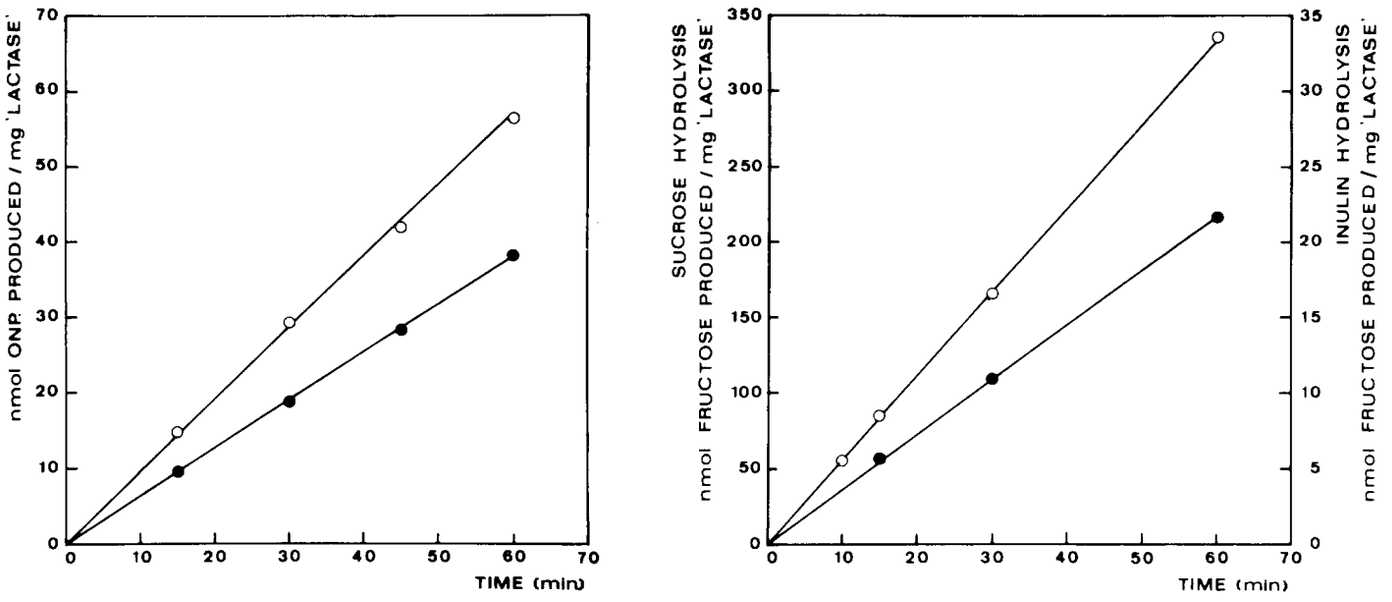

Fig. 4. **(left hand side)** *ONPG-hydrolysing activities of non-treated (●) and toluene-treated (○) aliquots of Beijerinck's lactase preparation. The assay was performed as described by Miller[9] at 30°C and pH 7.5. Cells were permeabilized by addition of toluene (final concentration 4% v/v) to a solution of 5 mg ml^{-1} lactase in 50 mM potassium phosphate buffer (pH 7.5) and incubation for 45 min at 30°C.* ***Fig. 5.*** **(right hand side)** *Sucrose (○) and inulin hydrolysis (●) by Beijerinck's lactase preparation. Activities were determined as described by Rouwenhorst* et al.[8].

cant lactase activity, linear both with time and enzyme concentration. Lactase activity increased when the preparation was first treated with toluene (Fig. 4), indicating that an additional amount of enzyme could be released from the yeast cells present in the preparation. Enzyme activities measured with lactose as a substrate were somewhat lower than those with ONPG (0.41 vs. 0.93 nmol of substrate hydrolysed per minute per milligram of the lactase preparation).

In his paper, Beijerinck reported the occurrence of sucrose-hydrolysing activity in lactase preparations. The *S.kefyr* strain originally isolated by Beijerinck has since been renamed *Kluyveromyces marxianus*. It is well known that *K. marxianus* produces an extracellular inulinase (EC 3.2.1.7) that is highly active towards sucrose[8]. It was to be expected that, if the lactase preparation had actually been obtained from Beijerinck's original *S. kefyr* culture, the preparation still might possess inulinase activity. Indeed, both sucrose- and inulin-hydrolysing activities could be measured in the lactase preparation (Fig. 5). The ratio of sucrose- to inulin-hydrolysing activity was about 16. This is entirely consistent with ratios observed with fresh *K. marxianus* inulinase preparations[8]. Wild-type *K. marxianus* produces a maximum inulinase activity of approximately 0.2 U mg^{-1} cells when grown in batch cultures on lactose. The 90-year old, dried lactase preparation contained approximately 0.005 U mg^{-1}. Thus, over a period of 90 years during which the preparation has been subjected to widely fluctuating temperatures, the inulinase activity probably had declined to a few per cent of its original activity.

Blessed are those who start now

In summary, our experiments indicate that the lactase prepared 90 years ago in Beijerinck's laboratory originated from a yeast. This yeast most probably was *S. kefyr*, the only yeast used by Beijerinck for research of lactose fermentation in that period. This is confirmed by the presence of both β-galactosidase and inulinase activity in the preparation. If the enzyme preparation described by Beijerinck in 1889 was prepared in the same way as the 1899 preparation described here, his crude enzyme preparations indeed contained lactase activity. However, Beijerinck's paper did not provide conclusive evidence for enzyme-catalysed lactose hydrolysis. Fischer[5] can be considered the real discoverer of lactase, since he was the first to prove beyond doubt that lactose hydrolysis can be catalysed by an enzyme. However, from Beijerinck's paper it is clear that he was the first to realize that an enzyme activity is involved in lactose hydrolysis. This perception represents just one of the many original ideas of a great scientist, whose work laid the foundation of modern microbiology.

After his retirement, Beijerinck once made the remark 'Gelukkig zij, die nu beginnen' ('Blessed are those, who start now'). The spectacular advances that have been made since and the fascinating problems still ahead demonstrate the relevance of this motto for those working in microbiology today.

Acknowledgement

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