PRODUCTION OF INULASE BY *CANDIDA KEFYR* AT SUB-OPTIMAL GROWTH TEMPERATURES

A thesis

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James Henry Bouch

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ABSTRACT

The time course production of inulase by Candida kefyr was investigated using sub-optimal growth temperatures. Studies indicated that inulase synthesis was directly contingent on biomass accumulation which directly influenced the rate of carbohydrate assimilation and as such, enzyme production. Both the intracellular and extracellular inulases appeared to be repressed by the presence of rapidly metabolizable carbon substrates. The catabolite repression of inulase observed in this investigation could not be altered by the addition of the surfactant (Tween 80). Addition of yeast extract (0.35% w/v) significantly promoted inulase production. Increasing aeration-agitation rates from 150 to 300 rev min^{-1} increased total inulase production. Distribution of intracellular and extracellular inulase as a consequence of sub-optimal growth could not be ascertained. Increasing the growth temperature from 10 to 30^{o}C and/or inoculum, increased inulase production.

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INTRODUCTION

Today, the sweetener which forms the main bulk of the sugar industry is sucrose. This disaccharide is the most abundant free sugar in the plant kingdom and is mainly refined from sugar cane and sugar beets. In recent years alternative sweeteners to sucrose have been investigated due to the unfavourable health aspects attributed to sucrose consumption. Present available literature reports indicate that sucrose has strong cariogenic properties and may also be involved as a factor in coronary diseases (Pilnik and Vervelde, 1976).

One of the possible alternative sweeteners being considered for human consumption and which has replaced sucrose to some extent is the monosaccharide fructose. An important nutritional aspect of fructose is that it is approximately 1.5 times sweeter than sucrose and therefore intake is reduced in terms of absolute amounts as well as in total calories. Other important factors include the fact that fructose is much less cariogenic and may be consumed by most diabetics. There are also industrial advantages favouring the use of fructose in the food industry such as its reduced tendency to crystallize, low viscosity, high hygroscopcity, high osmotic pressure and its fermentability (Fruin and Scallet, 1975). Inulin, a commonly occuring reserve carbohydrate found in plants belonging to the family Compositae has been shown to be an excellent source of fructose. Structurally, inulin consists of fructofuranose units that are $\beta(2\rightarrow1)$ linked

together in a non-branching chain, terminating in a $\alpha(2\rightarrow1)$ linked non-reducing D-glucose end group (Pilnik and Vervelde, 1976). Of the various methods investigated for the hydrolysis of inulin, it was determined that enzymatic action allowed for simpler purification of the final product and reduction of colour and flavour artifact induced by acid hydrolysis. Industrial application of enzymatic hydrolysis can only be feasible if the required enzyme is made available in large quantities at a competitive cost. Although parameters for inulase (β-fructofuranosindase) synthesis have been investigated using several microbial sources, the effects of sub-optimal temperature on enzyme production have not been intensively investigated. GrootWassink and Fleming (1980) reported that optimum temperature for growth and enzyme synthesis differed considerably. There are several instances where extracellular enzyme formation has been shown to be greater when the organism was grown at temperatures of lower than the optimum (Buehler et al., 1951; Peterson and Gunderson, 1960; Simpson, 1956). This study was initiated in order to evaluate inulase production at various sub-optimal growth temperatures in an attempt of increasing enzyme yield and/or altering the distribution of intracellular and extracellular inulase. This latter point was also considered important since extensive solubilization of the enzyme during fermentation presents recovery problems which could adversely affect the economics of enzyme procurement.

REVIEW OF LITERATURE

INULIN

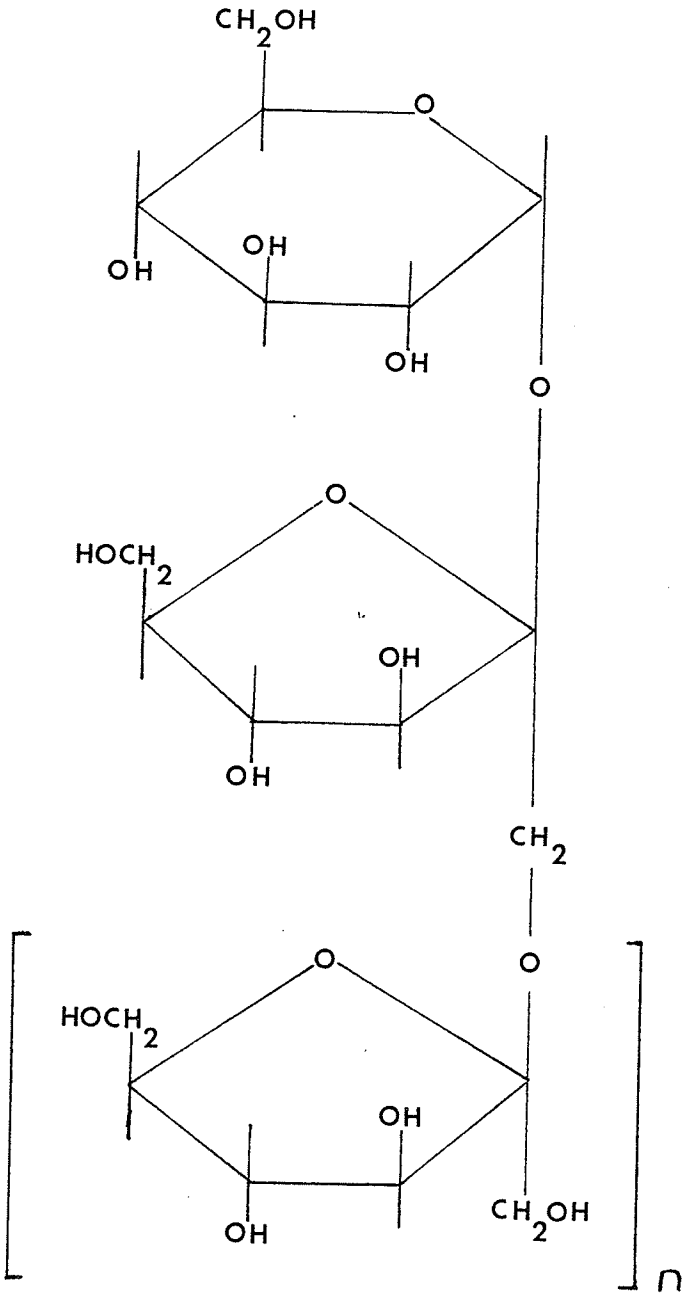
Structure

Structurally, inulin is a polymer consisting of fructofuranose sub-units linked together in a non-branching chain in which a non-reducing D-glucose forms the terminal end group (Fig. 1). The general formula is given by G∼F-Fn, where (G∼F) refers to the sucrosyl group which is $\alpha(2\rightarrow1)$ linked. The number of fructofuranose units (n) that are $\beta(2\rightarrow1)$ linked to each other may have a value anywhere from zero (sucrose) to approximately thirty-five (inulin) (Edelman and Jefford, 1968; Rutherford and Weston, 1968).

Physical and Chemical Properties

The average molecular weight of an inulin molecule is approximately 5,000 (Merck Index, 1976; Middleton, 1977; Percival, 1962). Based on physico-chemical data reported in the literature, Middleton (1977) proposed that the saturated inulin molecule is cylindrical with a semi-length of 25 Å and a radius of 10 Å. The configuration of the molecule was also suggested to be compact as opposed to extended as a result of the fructofuranose units being $\beta(2\rightarrow1)$ linked (Middleton, 1977). A steric model proposed by Phelps (1965) suggested that the inulin molecule is essentially a helical structure. There are disagreements in the reported literature on how many fructofuranose units exists per turn; approximately four units per turn have been proposed by Middleton (1977) and Phelps (1965).

FIGURE 1. Molecular Structure of Inulin.



Inulin in its purest state is described as being a white, amorphous powder. The solubility of inulin varies from being soluble, in hot water (3.91 g /100 g of water at 75°C) to slightly soluble in cold water (0.03 g /100 g of water at 15°C) (Phelps, 1965). In addition, low molecular weight polyfructans have been shown to have greater water solubility in comparison with higher molecular weight inulin-type polyfructans (Fleming and GrootWassink, 1979). The inulin molecule is also slightly soluble in diethyl ether and acetone and is insoluble in ethyl alcohol and benzene (Merck Index, 1976; Weast, 1980). Inulin has a melting point of 178°C at which temperature it undergoes decomposition (Weast, 1980).

Occurance and Use

Inulin being a polymeric fructosan is commonly found as a reserve carbohydrate in various plants belonging to the family Compositae (Aspinall, 1970; Percival, 1962). Some of the plants that have been reported to contain inulin include chicory (*Cichorium intybus*), dahlia tubers (*Dahlia sp.*), wild onion (*Allium sp.*), Canada thistle (*Cirsium arvense*), goldenrod (*Solidago sp.*), and Jerusalem artichoke (*Helianthus tuberosus*) (Chubey and Dorrell, 1974).

So far, developments for inulin usage have included commercial applications in the food and health fields. Healthwise, inulin has been applied as a marker used for the determination of glomerular filtration rates in renal physiology studies (Middleton, 1975; Olbight *et al.*, 1977; Phelps, 1965). Jerusalem artichoke and chicory roots have also been used as an inulin source to successfully

produce industrial grade alcohol via microbial fermentation (Artegas and Ayzela, 1944; Haken, 1942; Underkofler et al., 1937). In Canada, the Jerusalem artichoke has primarily been investigated as a possible source for inulin, largely because of its high inulin content per se and its ability to grow in less fertile land (Chubey and Dorrell, 1974; Pilnik and Vervelde, 1976). In recent years there has been a surge of renewed interest in research with inulin, specifically, the hydrolysis of inulin to fructose as an alternative sweetener.

INULIN HYDROLYSIS

Research reported in the literature referring to inulin and fructan hydrolysis has involved the application of enzymatic hydrolysis and various means of acidifying the artichoke extract. Enzymatic hydrolysis of inulin by an enzyme inulase has been thoroughly investigated (Avigad and Bauer, 1966; Beluche et al., 1980; Fleming and GrootWassink, 1979). Inulin hydrolysis by means of acidification has mainly dealt with the addition of acids such as hydrochloric, sulfuric, citric and phosphoric to facilitate inulin hydrolysis (Dykins et al., 1933; Eichinger et al., 1932; Fleming and GrootWassink, 1979). Other means of acidification that have received some attention include the use of ion exchange (Englis and Fiess, 1942; Fleming and GrootWassink, 1979; Kierstan, 1978) and electrodialysis (Dykins et al., 1933; Hardy, 1933).

Ion Exchange

Inulin hydrolysis studies involving the application of ion exchange resins have been reported to produce a clear, palatable, fructose syrup from Jerusalem artichoke and chicory extracts (Englis and Fiess, 1942; Kierstan, 1978).

Kierstan (1978) reported that by using a strong cation exchanger (H^+ form) the pH of the extract could be lowered to ≤ 2 in order to facilitate inulin hydrolysis. This was accomplished by the ability of the resin to replace the naturally occuring cations in the extract with hydrogen ions of the exchanger (Englis and Fiess, 1942; Fleming and GrootWassink, 1979). As a supplementary treatment, the

hydrolyzate was neutralized to a pH 6-7 by the use of an anion exchange resin (Englis and Fiess 1942; Kierstan, 1978). Kierstan (1978) examined a number of possible exchange resins. From his study, most consideration was given towards the application of Amberlite IR-120 and Amberlite IR-A93 as cation and anion exchange resins, respectively.

Favourable aspects attributed to the use of ion exchange resins included effective salt removal, total hydrolysis, reduction of coloured matter and minimal loss of product (Englis and Fiess, 1942; Kierstan, 1978). However, substantial capital outlay for recycling equipment was shown to render this method unsuitable for high fructose syrup production (Fleming and GrootWassink, 1979).

Electrodialysis

Successful hydrolysis of Jerusalem artichoke extracts through the use of electrodialysis has been reported by Fleming and GrootWassink (1979) and Hardy (1933). The acidification of the extract in this method was accomplished by the selective removal of anions under the influence of an electrical potential. The principle drawback of electrodialysis, however, is that it has not been applied in a full scale continuous food processing system (Fleming and GrootWassink, 1979).

Acid Hydrolysis

Acid hydrolysis studies reported to date have mainly been concerned with optimizing the conditions of inulin hydrolysis concomitant with the minimal destruction of fructose.

The rate of inulin hydrolysis was shown to be primarily a function of pH with time; temperature contributed as a secondary factor (Dykins and Englis, 1933). Much of the work performed on inulin hydrolysis has involved the utilization of inulin containing Jerusalem artichoke extracts. The high buffering capacity of the artichoke extract has made it necessary to measure the available acidity using pH rather than normality (Dykins and Englis, 1933; Eichinger et al., 1932; Fleming and GrootWassink, 1979). It has also been shown by Dykins and Englis (1933) that the normality of the acid required to reach a desired pH increased with the percentage of total solids contained within the extract. Various acids have been used to lower the pH of the extract; Fleming and GrootWassink (1979) stated that there were no apparent differences in the effectiveness of the various acids (hydrochloric, citric and sulfuric; pH 2) towards fructan hydrolysis. The degree of polymerization (DP) of the polyfructosan was shown to have little effect on the hydrolytic conditions required with regards to hydrolyzing the $\beta(2\rightarrow1)$ furanosidic linkages. Alternatively, the $\alpha(2\rightarrow1)$ linked fructofuranose units required harsher hydrolyzing conditions (Fleming and GrootWassink, 1979). Studies have pointed out that fructose degradation is a common occurence under thermal and acidic conditions forming undesirable off-flavours and coloured compounds (Fleming and GrootWassink, 1979; Shaw et al., 1967).

Many researchers have attempted to optimize the conditions used for acid hydrolysis. Much of the concern has

centered with achieving a favourable rate of hydrolysis concomitant with the formation of an acceptable syrup. McGlumphy et al. (1931) using a semi-commercial operation utilizing artichoke extracts (40% total solids) tried to obtain total hydrolysis within one hour after acidification.. The extract was acidified to a pH of 1.5 with sulfuric acid and heated to 80°C for one hour. Bucke (1979) reported that similar conditions were utilized using a lower temperature (75°C). Complete hydrolysis was reported after acidification of the inulin-containing extracts containing 40% total solids with hydrochloric acid (pH 1.75) when heated for one hour at 80°C (McGlumphy et al., 1941). Dykins and Englis (1933) employed a higher pH in which the artichoke extracts were subjected to a pH of 4.2 (hydrochloric acid) for twenty minutes at 130°C. Milder hydrolytic conditions employing a pH 3 to 4 with temperatures of 70 - 80°C have yielded 80 - 100% hydrolysis with the formation of a superior final product (Fleming and GrootWassink, 1979).

The removal of acid from the inulin extract after hydrolysis is refered to as neutralization. The technological difficulty of acid removal has often contributed to the cost of the acid hydrolysis process (Fleming and GrootWassink, 1979). Acids used to acidify artichoke extracts have been classified into two main groups. One group includes those acids whose anions can be complexed during neutralization to form an insoluble precipitate. The insoluble salts formed can then be removed by filtration. Difficulty has

been encountered, however, on the concentration of the dilute syrup, afterwhich an additional unwanted precipitate was formed over a period of time (Dykins and Englis, 1933). Neutralization of sulfuric and phosphoric acid have been accomplished through the use of sodium hydroxide or carbonates of calcium, barium or strontium (Dykins et al., 1933). Citric and oxalic acids have been reported to form calcium citrate and calcium oxalate, respectively, when neutralized with calcium oxide (Fleming and GrootWassink, 1979). Excess calcium was subsequently precipitated as calcium carbonate. Acidified extracts neutralized with calcium hydroxide have been reported to have a bitter flavour (Dykins et al., 1933). This flavour was attributed to the possible presence of calcium ions in the syrup.

The second group of acids consists of those whose anions remain essentially in the hydrolyzed extract mainly as soluble sodium salts such as hydrochloric acid (Dykins et al., 1933). Neutralization of the acidified extract with either sodium hydroxide or sodium carbonate resulted in the formation of sodium chloride which contributed to a salty taste and increased ash content in the finished syrup (Dykins et al., 1933; Flening and GrootWassink, 1979).

The harshness of the hydrolytic conditions is thought to be responsible for affecting the final quality of the syrup. Reports indicate that acid-catalyzed degradation reactions involving fructose, formed off-flavoured and coloured compounds through a combination of enolization and

dehydration processes (Shaw et al., 1967). A comparison study by Fleming and GrootWassink (1979) investigated the development of colour and off-flavours in artichoke syrups under conditions of high (pH 1.5) and low (pH 3.5) acidity. The studies showed that highly acidic conditions formed a syrup with lower levels of colour but with a significantly greater development of off-flavours as compared with syrups formed at a lower acidity. Any attempts to deal with the purification of the syrup at this stage was shown to result in sugar loss (Fleming and GrootWassink, 1979). Due to potentially high processing costs and poor quality of the final syrup, enzymatic hydrolysis of inulin was turned to as an alternative means of inulin hydrolysis.

Enzymatic Hydrolysis

Fleming and GrootWassink (1979) reported enzymatic hydrolysis to be a more favourable approach to artichoke extract hydrolysis as opposed to acidic methods. Enzymatic hydrolysis of tuber extracts was shown to yield a syrup with minimal colour and flavour development. Inulin and lower molecular weight fructosans are both composed of β-2, 1-fructofuranosidic linkages, and have been shown to be hydrolyzed by a group of identified fructofuranosidases (Beluche et al., 1980; Fleming and GrootWassink, 1979). This group of enzymes was differentiated by their specificity towards inulin and sucrose. An enzyme with a high hydrolytic activity towards inulin has been denoted as an inulase or systematically named β-2, 1-fructan fructanohydrolase [(EC 3.2.1.7) IUBC, 1965]. Alternatively, invertase is a β-fructofuranosidase with similar enzymatic action as inulase but is more specific towards sucrose (Nahm and Byun, 1977). The generic name for this enzyme is β-D-fructofuranosidase fructohydrolase [(EC 3.2.1.26) IUBC, 1965]. The fructofuranosidases are characterized by an activity ratio as determined by the hydrolysis rates of sucrose to inulin (S/I ratio). S/I ratios of 14,000 and 16,666 have been reported for the invertase produced by Saccharomyces cerevisiae (Negoro and Kito, 1973[b]; Snyder and Phaff, 1960). Studies using inulase on the other hand have indicated much lower S/I ratios ranging from 0.78 - 78.8 as shown in Table 1. The rate of inulin hydrolysis by inulase is a more favoured reaction than that of sucrose hydrolysis

TABLE 1. The S/I Ratio of Inulases Isolated from Various Microorganisms.

	Organism	S/I Ratio
GrootWassink and Fleming (1980)	S. fragilis ATCC 12424	10.5
Guiraud et al. (1980)	C. salmenticensis	78.8
Nakamura and Hoashi (1969)	A. niger	4.5
	Penicillium sp.	0.87
Negoro (1978)	S. fragilis strain #541	28.5
Negoro (1973)	C. kefyr extracellular	46.6
Negoro and Kito (1973[b])	C. kefyr intracellular	41.7
Snyder and Phaff (1960)	S. fragilis strain #351	25.0
Takahashi and Soutome (1975[b])	L. plantarum F-101	1.0
	S. fragilis IAM 4763	43.0
	A. awamori	10.0
Yurkevich and Kovaleva (1972[a])	S. lactis	3.5

(Snyder and Phaff, 1960; Takahashi and Soutome, 1975[b]).

INULASE SOURCES AND PROPERTIES

Plants

Plant sources of inulase which have been examined include Jerusalem artichoke, chicory roots and the common dandelion (Edelman and Jefford, 1964; Flood et al., 1967; Rutherford and Deacon, 1972[a]). The β-fructofuranosidases isolated from these plant sources have been considered to be very similar in nature despite reported differences in hydrolysis rates (Rutherford and Deacon, 1972[a]). The rates of inulase hydrolysis has been shown to be highly dependent on the degree of polymerization (DP) of the inulin series. Edelman and Jefford (1964) showed that the rate of fructosan hydrolysis was insignificant for DP 2 but increased to an optimum for chain lengths of DP 5 - 8. The optimum hydrolysis rate for DP 5 - 8 was over twice the rate as compared to inulin (DP 30) hydrolysis.

The inulase found in the Jerusalem artichoke is considered to be a true or specific inulase. This particular inulase type is highly specific towards the β-2, 1 inulin-like linkages and is practically inactive towards sucrose (Avigad and Bauer, 1966; Edelman and Jefford, 1964). Similarly, β-fructofuranosidases isolated from the roots of dandelions were shown to be inactive towards sucrose (Rutherford and Deacon, 1972[b]). Artichoke isolated inulases were also found to be incapable of hydrolyzing both levan (β-2, 6 linkages) and irisin (mixed β-2, 1 and β-2, 6 linkages)

in enzyme specificity studies carried out by Avigad and Bauer (1966). Inulase isolated from both artichoke and dandelion were shown to have similar modes of action on inulin. Inulin degradation occured via an exo-type action with a stepwise release of monomeric fructose units from the non-reducing end of the chain (Edelman and Jefford, 1968; Rutherford and Deacon, 1972[b]).

The relative activity and stability of inulase isolated from artichoke tubers was shown to have an optimum pH range of 5.0 - 5.5 (Avigad and Bauer, 1966; Edelman and Jefford, 1964). Rutherford and Deacon (1972[a]) studied inulase activity from roots of dandelion using a phosphate-citrate buffer and reported a pH optimum of 4.0. Edelman and Jefford (1964) reported that inulase activity from artichoke tubers were partially inhibited in the presence of 10 mM concentrations of sucrose, 1^F-fructosylsucrose, inulobiose, raffinose and melezitose. Sucrose and 1^F-fructosylsucrose impeded inulin hydrolysis to the greatest extent causing 66.5% and 83.5% inhibition of inulase activity, respectively. Avigad and Bauer (1966) reported sucrose to be a noncompetitive inhibitor of inulin hydrolysis. Assay studies using dandelion inulase showed that the presence or absence of sucrose did not significantly inhibit inulase activity (Rutherford and Deacon, 1972[b]). Sugars which were not found to be inhibitory towards artichoke inulase at the 10 mM level were fructose, glucose, melibiose, maltose, 6-fructosylglucose, lactose and trehalose (Avigad and Bauer, 1966).

Since artichoke tubers have been shown to contain a low and somewhat variable inulase level, artichokes are not considered to be a good source of inulase (Fleming and GrootWassink, 1979).

Bacteria

There has been some attention directed towards characterization of bacterial inulase sources in past literature reports. The bacteria which have been studied in some detail include: Erwinia phytophthorum, Lactobacillus plantarum F-101 and Arthrobacter ureafaciens (Takahashi and Soutome, 1975[b]; Tolba and Ghanem, 1959; Tanaka et al., 1972). The soft rot bacterium E. phytophthorum has been reported to produce an intracellular inulase (Tolba and Ghanem, 1959). The results also indicated that the inulase produced by this bacterium is a constitutive enzyme. It was found that continued subculturing of E. phytophthorum in the presence or absence of inulin did not affect the rate of inulin hydrolysis by the organism. The rate of inulin hydrolysis occured at a much faster rate than with sucrose (Tolba and Ghanem, 1959) thereby substantiating the suggestion that the microorganism hydrolyzed inulin by a specific inulase; the S/I value was not, however, reported. The addition of 5% fructose into a basal medium containing 2% inulin was reported by these authors to display a partial reduction in terms of inulin utilization by E. phytophthorum. The reduced utilization rate of inulin was attributed to greater availability of fructose and not to inhibitory action by the monosaccharide.

Takahashi and Soutome (1975[b]) investigated inulase activity associated with L. plantarum F-101. The inulase was reportedly capable of hydrolyzing the substrates inulin, sucrose and raffinose. At the optimum pH, the relative enzyme activity towards the substrates sucrose and inulin was shown to be greater than with raffinose (Takahashi and Soutome, 1975[b]). Takahashi and Soutome (1975[b]) purified the extracellular β-fructofuranosidase contained within the culture filtrate using DEAE-Sephadex A-25 column chromatography. The specific activity of the purified enzyme preparation showed an increased specific activity, 200 times greater than the original unpurified culture filtrate. The study indicated that the purified inulase had a S/I ratio of 1 regardless of the reaction pH values. It was determined that a pH of 5.5 was optimum for maximum activity. This pH of 5.5 was also shown to have the greatest stabilizing effect on enzyme activity after the enzyme was incubated at 50°C for twenty minutes; the enzyme retained 75 - 80% of its original acitivity using assay conditions of 40°C for 20 minutes at pH 5.5 (Takahashi and Soutome, 1975[b]). Approximately 25 - 30% of the original enzyme activity remained when adjusted to pH 4.5. The purified inulase system remained relatively stable at 50°C, at higher temperatures, thermal inactivation of the enzyme was greatly accelerated. At 60°C complete inactivation of β-fructofuranosidase activity occured after 5 minutes.

Takahashi and Soutome (1975[b]) studied the effects of metal ions (10^{-4} M) and a sulfhydryl (SH) reagent (p-chloromercuribenzoate) on β-fructofuranosidase activity. The enzyme activity was completely inhibited by the presence of Hg^{2+} and Ag^{+} metal ions in the enzyme mixture. Inhibition of enzyme activity by Cu^{2+} and Pb^{2+} was 36% and 51%, respectively. Alternatively, the presence of Ba^{2+}, Ca^{2+}, Co^{2+}, CN^{-}, Mg^{2+}, Ni^{2+}, Mn^{2+}, and Zn^{2+} caused little or no inhibition of inulase activity. The SH reagent at a 5×10^{-6} M concentration resulted in 86% inhibition of activity. The β-fructofuranosidase reactivity towards sucrose was demonstrated to be competitively inhibited by the addition of 5% inulin (Takahashi and Soutome, 1975[b]). Tanaka et al. (1972) reported that A. ureafaciens produced an extracellular enzyme designated as inulase II. The main reaction products from the enzymatic action of inulase II on inulin was shown to be difructose anhydrides along with small residual amounts of oligosaccharides. The difructose anhydrides were identified as being a di-D-fructofuranose 1,2':2,3' dianhydride (Uchiyama et al., 1973). It was later determined that inulase II was an exo-enzyme acting from the non-reducing end of inulin (Uchiyama, 1975). Inulase II was shown to differ from β-2, 1-fructan fructanohydrolase (EC 3.2.1.7) which normally hydrolyzes individual fructose units from the non-reducing end of the inulin molecule (Avigad and Bauer, 1966; Snyder and Phaff, 1962). Enzymatic properties were examined using a purified inulase II preparation by Uchiyama et al. (1973). The enzyme was shown to remain stable within a wide pH range of 4 to 11. Inulase II

was reported to be optimally reactive at pH 6 and at a temperature of 50°C. Thermal stability studies indicated that the enzyme remained relatively stable at incubation temperatures between 20 to 50°C. Rapid inactivation of the enzyme occured with temperatures above 60°C. Inhibitory effects were demonstrated with the presence of the heavy metal salts, $HgCl_2$ and $PbCl_2$ at 1 mM concentrations. Uchiyama et al. (1973) reported that the divalent cations ($MgCl_2$, $CaCl_2$, $MnCl_2$, $ZnCl_2$); EDTA and cysteine had little or no effect on inulase II activity.

Molds

Reports on inulase production have indicated that several molds are capable of producing a β-fructofuranosidase enzyme. Molds that have been identified as potential inulase sources are Aspergillus awamori, Penicillium sp. and Aspergillus niger (Kim, 1975; Nakamura and Hoashi, 1969; Takahashi and Soutome, 1975[a]). Takahashi and Soutome (1975[a]) demonstrated that inulase produced by A. awamori was able to hydrolyze inulin, sucrose and raffinose. The ratio of hydrolysis rates for sucrose and inulin (S/I ratio) was established to be 10. This would indicate that the inulase produced by A. awamori could be classified as a non-specific inulase. The pH optimum for maximum β-fructofuranosidase activity was determined by Takahashi and Soutome (1975[a]) to be 4.5. This pH value was also found to be the same for the hydrolysis of inulin, sucrose and raffinose. These researchers also demonstrated that both inulin and raffinose competitively inhibited the sucrose hydrolyzing

ability of the β-fructofuranosidase.

The inulin producing ability of Penicillium sp. was examined by Kim (1975) and Nakamura and Hoashi (1969). A S/I ratio of 0.87 was reported.

Nakamura et al.(1978[a]) reported the production of an extracellular inulase by A. niger when cultured in an inulin and corn steep liquor based medium. The enzyme showed a high specificity towards inulin and was denoted as inulase (I). The isolated inulase was shown to hydrolyze inulin but not sucrose or raffinose. Analysis of end products derived from inulin hydrolysis was also performed. The results indicated that inulotriose was formed in greater quantities followed by inulotetraose and inulopentaose, respectively. It was therefore evident that the pattern of enzymatic action by inulase (I) was of an endo-type action. The unpurified enzyme was isolated from the culture filtrate in crystalline form by use of Sephadex column chromatography and by $(NH_4)_2SO_4$ fractionation (Nakamura et al., 1978[b]). Characterization studies with the isolated inulase (I) showed that a temperature of 45°C and a pH of 5.3 were optimum for maximum enzyme activity. It was also determined that the divalent metal ion, Mn^{2+} served as an activator for inulase activity. Enzyme inhibition was noted in the presence of Ag^{+}, Hg^{2+} and p-chloromercuribenzoate.

Yeasts

The culturing of a yeast inulase source has been investigated by numerous workers. GrootWassink and Fleming

(1980) indicated that in a commercial process a yeast would be more appropriate as opposed to other inulase producing microorganisms. Inulase producing yeasts which have been studied in some detail include: Saccharomyces lactis, Debaromyces cantarellii, Candida salmenticensis, Saccharomyces (Kluveromyces) fragilis and Candida kefyr (Beluche et al., 1980; Guiraud et al., 1980; Nahm and Byun, 1977; Negoro and Kito, 1973[a]). There is a disagreement in classifying the enzymatic activity as to whether an inulase or an invertase is synthesized by the yeasts. Yurkevich and Kovaleva (1972[a]) working with purified β-fructosidase preparations from several yeasts concluded that an invertase was responsible for the hydrolysis of both sucrose and inulin. This reasoning was based on S/I values where the rate of sucrose hydrolyzed surpassed the rate of inulin hydrolysis such as in the case of 3.5 for S. lactis. However, in many cases it was generally accepted that inulase was responsible for the hydrolysis of sucrose and inulin by yeasts (Negoro and Kito, 1973[a]; Snyder and Phaff, 1960). In these instances, the enzymes were classified as inulases after determining that the rates of inulin and sucrose hydrolysis were comparable (Fleming and GrootWassink, 1979). This latter classification of inulase was used throughout this thesis. Inulase purification was accomplished using DEAE cellulose chromatography and polyacrylamide gelelectrophoresis (Fleming and GrootWassink, 1979; Negoro and Kito. 1973[b]). The results demonstrated that the enzyme, inulase, possessed both inulin and sucrose hydrolysis activities. Yurkevich and Kovaleva (1972[b]) using competitive inhibition

studies determined that both enzyme activities involved the same active site on the enzyme.

The location site for inulase in D. cantarellii and C. salmenticensis was examined by Beluche et al. (1980) and Guiraud et al. (1980). The yeast cells were homogenized and the resultant extracts were fractionated by centrifugation. Experimental results showed that inulase activity was present both within the cell wall fraction and in the supernatant. The inulase enzyme in yeasts was thought to be bound to the cell wall since whole or ruptured protoplasts lacked inulase activity (Beluche et al., 1980). Guiraud et al.(1980) reported that crushing whole cells accounted for the higher level of inulase activity in the supernatant (3.5 µm fructose per min. per mg. protein) in comparison to 0.91 for the cell wall fraction. The inulase was considered to be weakly bound to the cell wall and could easily be extracted (Beluche et al., 1980). Extraction of the cell wall enzyme from D. cantarellii was accomplished by treating whole cells with 0.02 M phosphate buffer at pH 8.5, 20°C. After three extractions the inulase activity was reported to be 210% of the initial cell activity. The culturing of D. cantarellii in a yeast nitrogen base medium with 0.5% inulin and 0.02 M phosphate buffer was examined by Beluche et al. (1980). The results indicated that during the stationary phase, the culture medium contained 5 times more inulase activity than that of intact cells. Normally, culturing D. cantarellii in the absence of phosphate buffer resulted in no activity in the culture medium. Beluche et al. (1980) therefore suggested that the

composition of the medium may effect the excretion of the cell wall enzyme. Guiraud et al. (1980) studying inulase production by C. salmenticensis found that inulase was released by the lysis of the cell wall during the time of protoplast formation.

Some interest has been generated towards the yeasts C. salmenticensis, D. cantarellii and S. lactis in terms of inulase production and characterization. The inulase produced by C. salmenticensis and D. cantarellii has been shown to hydrolyze inulin, sucrose and raffinose (Beluche et al., 1980; Guiraud et al., 1980) while S. lactis was found to hydrolyze both inulin and sucrose (Yurkevich and Kovaleva, 1972[a]). The inducible nature of inulase has been demonstrated in C. salmenticensis and D. cantarellii (Beluche et al., 1980; Guiraud et al., 1980). Inulase production by both yeasts was induced by culturing them in an inulin containing medium. In addition, sucrose and raffinose were also shown to be inducers of inulase activity in C. salmenticensis. Guiraud et al. (1980) demonstrated that inulin as a carbon source, had the greatest effect on the synthesis of extracellular inulase production followed by the substrates raffinose and sucrose, respectively. Alternatively, inulase induction did not occur with D. cantarellii in the presence of the substrates raffinose, sucrose, glucose and fructose (Beluche et al., 1980). The enzymatic action of the inulase synthesized by C. salmenticensis and D. cantarellii has been studied by both Guiraud et al. (1980) and Beluche et al. (1980). It

was determined that inulase from these sources hydrolyzed inulin in a stepwise fashion beginning from the non-reducing end of the inulin molecule. Glucose appeared at the end of the hydrolysis reaction.

A homogeneous inulase preparation from S. lactis utilizing DEAE-Sephadex A-50 chromatography exhibited a S/I coefficient of 3.5 (Yurkevich and Kovaleva, 1972[a]). The S/I value obtained from the C. salmenticensis cell wall fraction has been reported to be 78.8 (Guiraud et al., 1980). The effect of pH on inulase activity from D. cantarellii was examined by Beluche et al. (1980). The cell wall enzyme was extracted with a phosphate buffer to produce a relatively pure enzyme extract after dialysis. The results reported a pH optimum of 4.0 for the substrates inulin, sucrose and raffinose. In conjunction with these findings, a minor pH optimum of 6.0 was observed for the substrates sucrose and raffinose. It was therefore suggested by Beluche et al. (1980) that the presence of two enzymes might exist. Inulase activity from C. salmenticensis was found to have a pH optimum of 4.0 for the substrates raffinose and sucrose; (Guiraud et al., 1980) a lower pH range of 3.5 - 4.0 for inulin was demonstrated. Optimum pH values for S. lactis inulase activity on sucrose and inulin, however, were 3.3 and 4.1, respectively (Yurkevich and Kovaleva, 1972[a]). These authors also reported that a single enzyme could have different pH optima depending on the specific substrates available. The effect of temperature on inulase activity from D. cantarellii and C. salmenticensis on inulin hydrolysis was also studied. The optimum temperature

for inulase activity was shown to be 40 - 45°C for D. cantarellii and 46°C for C. salmenticensis at pH 4.0 (Beluche et al., 1980; Guiraud et al., 1980). Optimal temperature for the hydrolysis of raffinose and sucrose were 52°C and 60°C, respectively, using a purified inulase from C. salmenticensis (Guiraud et al., 1980). S. lactis inulase optimally hydrolyzed both sucrose and inulin at 37°C (Yurkevich and Kovaleva, 1972[a]). Thermal stability studies performed with D. cantarellii inulase showed a 66% loss in enzyme activity after 30 minutes incubation at 35°C (Beluche et al., 1980). The enzyme displayed total inactivation when incubated for a period of 10 minutes at 60°C. The inulase synthesized by C. salmenticensis, however, was reported to be relatively stable at 50°C (Guiraud et al., 1980). Additional work by these reporters demonstrated that thermal inactivation firstly effected the hydrolysis of inulin, followed by raffinose and then sucrose. Only residual activity towards raffinose and sucrose was obtained when the enzyme was incubated at 55°C for 25 minutes; incubation at 60°C for 10 to 15 minutes, however, resulted in trace activity towards sucrose. No activity for inulin and raffinose was detected. Guiraud et al. (1980) considered that these results suggested a progressive modification of the enzyme's active site, thereby effecting the steric nature of the active site. Low temperature effects on inulase isolated from D. cantarellii were investigated by Beluche et al. (1980). Storage of the enzyme preparation at 4°C resulted in a 50% loss and total loss in activity when stored for a time period of 25 days and 3 months, respectively.

Storage of the frozen enzyme extract rendered an active preparation after several months. Strong inhibitory action (83 - 100%) towards the inulase activity from C. salmenticensis was demonstrated by the presence of 1×10^{-3} M of $PbNO_3$, $AgNO_3$, I_2, and HgCl (Guiraud et al., 1980). Lower levels of inhibition (50 - 62%) were shown to occur with $FeCl_2$, acetic anhydride and N-bromosuccinimide (1×10^{-3} M). Inhibitory effects by these agents on inulase was shown to be greater when in the presence of inulin as opposed to raffinose followed by sucrose (Guiraud et al., 1980). The inulase from D. cantarellii was inhibited by 50 - 85% in the presence of $HgCl_2$, $AgNO_3$, $Pb(NO_3)_2$, 2-mercaptoethanol and N-bromosuccinimide at 1×10^{-3} M (Beluche et al.,1980). Also, total inhibition of inulase activity was found to occur with 1×10^{-2} M $FeCl_3$. Inulases obtained from D. cantarellii and C. salmenticensis were reported not to require the presence of any metal activators or co-factors of low molecular weight (Beluche et al., 1980; Guiraud et al., 1980).

Among the inulase producing yeasts, several strains of Saccharomyces fragilis have been examined. An intracellular and extracellular inulase was shown to be synthesized by S. fragilis strain No. 351 when cultured in an inulin containing medium by Nahm and Byun (1977) and Snyder and Phaff (1960). Similarly, S. fragilis strain No. 0541 and American Type Culture Collection (ATCC) 12424 were reported by Negoro (1978) and GrootWassink and Fleming (1980), respectively, to produce both an intracellular and extracellular inulase. Snyder and Phaff (1960) reported that the inulase enzyme was

induced to its highest yield with inulin as the carbon source and to a lesser degree with raffinose. Poor inducers of inulase were found to be fructose, sucrose and glucose. A purified inulase preparation was shown to actively hydrolyze sucrose, raffinose and inulin (Negoro, 1978; Snyder and Phaff, 1962). Since complete hydrolysis of bacterial levans by yeast inulase was demonstrated by Snyder and Phaff (1960), it was concluded that the inulase isolated from S. fragilis was capable of hydrolyzing both β 2→1 and β 2→6 fructofuranosidic linkages. In addition, there were no observable differences in the relative activities between the intracellular and extracellular inulase towards inulin, raffinose and sucrose (Negoro, 1978). Both the intracellular and extracellular inulase sequentially hydrolyzed individual fructose units from the non-reducing end of the inulin molecule (Avigad and Bauer, 1966). Nahm and Byun (1977) reported that as inulin hydrolysis progressed, increased quantities of fructose were formed corresponding with the appearance of lower molecular weight oligosaccharides. Fructose was reported to be the principle sugar formed during the hydrolysis reaction and also a small quantity of glucose was detected in the latter stages of reaction. The trisaccharide, melezitose, (3-O-α-D-glucopyranosyl-β-D-fructofuranosyl-α-D-glucopyranoside) was not hydrolyzed by inulase supporting the exo-type action of the S. fragilis inulase (Negoro, 1978; Snyder and Phaff, 1962). Melezitose contains the identical terminal configuration as the inulin molecule (Snyder and Phaff, 1962). However, the centrally positioned fructosyl residue is protected by the second glucosyl bonded

to the fructose in the 3-position. Negoro (1978) reported no α-glucosidase activity at pH values of 3 - 7. Degradation of inulin by inulase occured by a single-chain mechanism (Nahm and Byun, 1977; Snyder and Phaff, 1962). Experimental results demonstrated that the enzyme entirely hydrolyzed an inulin molecule one at a time (single-chain mechanism) compared to the enzymatic hydrolysis of fructose end units from inulin chains at random. This latter type of hydrolysis is referred to as a multi-chain mechanism. The rate of glucose appearance, in relation to the increase in total reducing sugars during the hydrolysis, provided some information regarding the mechanism-type. The fructose to glucose ratio for 0.01 N H_2SO_4 hydrolyzed inulin was 35.1 (Snyder and Phaff, 1962). Enzymatic hydrolysis of inulin approached similar values at conditions of pH 5.1 at 30°C (Snyder and Phaff, 1962). Nahm and Byun (1977) reported that at pH 5.0 (50°C) a lower fructose to glucose ratio resulted (23:1). This difference was attributed to the possible lower degree of polymerization in the standard inulin used. Initial stages of the reaction showed high fructose to glucose ratio values which were explained by the need for an absolute number of fructose molecules to be hydrolyzed before the appearance of free glucose (Snyder and Phaff, 1962). The early detection of free glucose resulting from the hydrolysis reaction further demonstrated the presence of a single-type chain mechanism which was further investigated under varying conditions of pH and temperatures (Snyder and Phaff, 1962). Essentially, there was no alteration from the single-type

mechanism observed at pH 5.1 (optimum) at temperatures of 50°C and 0.5°C or at pH 7.0 at 30°C. However, at pH 3.0 (30°C) there was a notable shift towards a multi-chain mechanism. This was verified by a fructose to glucose ratio greater than 35, signifying a delayed appearance of glucose during hydrolysis.

The range of S/I values reported for S. fragilis purified inulase include 10.5 (GrootWassink and Fleming, 1980), 28.5 (Negoro, 1978), and 25.0 (Snyder and Phaff, 1960). Snyder and Phaff (1960) indicated that there were no significant differences in S/I values for intracellular and extracellular inulase preparations. The ratio of hydrolysis rates with raffinose and inulin (R/I) were found to be 3.5 and 4.1 (Negoro, 1978; Snyder and Phaff, 1960). Reports indicated optimum pH ranges of 3.0 - 4.3 for sucrose hydrolysis and 5.0 - 5.5 for inulin hydrolysis (Nahm and Byun, 1977; Snyder and Phaff, 1960; Yurkevich and Kovaleva, 1972[b]). A lower pH optimum of 4.5 was reported for inulin hydrolysis by Negoro (1978). The pH optimum and pH activity curves showed little variation between the intracellular and extracellular inulase isolated from S. fragilis (Negoro, 1978; Snyder and Phaff, 1960). The enzyme activity was stable between pH 2.0 - 8.0 with a loss of activity occuring only at a pH greater than 8.5 (Negoro, 1978). Nahm and Byun (1977) and Negoro (1978) both determined that maximal inulin hydrolysis by a purified inulase from S. fragilis occured at 55°C. This optimal temperature was identical for both intracellular and extracellular inulase preparations (Negoro, 1978). A

temperature of 60°C, however, was reported for the optimum hydrolysis of sucrose by inulase (Nahm and Byun, 1977). Temperature inactivation studies by Negoro (1978) and Snyder and Phaff (1960) demonstrated temperatures greater than 60°C caused a loss in enzyme activity towards sucrose while Nahm and Byun (1977) reported a near total loss in enzyme activity within 15 minutes at temperatures over 65°C. The ratio of hydrolysis rates of sucrose to inulin remained relatively constant during the partial inactivation of the enzyme by heat (Negoro, 1978; Snyder and Phaff, 1960). It was therefore suggested that both sucrose and inulin hydrolysis occur via a single enzyme or that the enzymes involved are inactivated at the same rate (Negoro, 1978). The sucrose hydrolyzing ability of the inulase preparation isolated by Nahm and Byun (1977), however, was more susceptible to temperature denaturation as compared to its inulin hydrolyzing activity. Reports have indicated that both intracellular and extracellular inulase show similar rates of temperature denaturation (Negoro, 1978; Snyder and Phaff, 1960). The thermostability of inulase was shown to increase up to a temperature of 70°C in the presence of high sucrose concentrations (GrootWassink and Eleming, 1980). Yurkevich and Kovaleva (1972[b]) determined that pyridoxal and pyridoxin both served as inulase activators and that pyridoxin did not alter the ratio of the reaction rates between sucrose and inulin hydrolysis. Both intracellular and extracellular inulase were reported by Negoro (1978) to be partially inhibited by

mercuric chloride, phenylhydrazine, silver nitrate and p-chloromercuribenzoate. Inhibitory action by aniline on inulase activity was reported by Nahm and Byun (1977). Inhibitory action of sodium dodecyl sulfate, urea and guanidine was more evident towards intracellular inulase as opposed to extracellular inulase (Negoro, 1978). Yurkevich and Kovaleva (1972^{b}) also reported that an increased substrate concentration of either sucrose or inulin invariably led to a reduced level of enzyme activity; a competitive relationship was shown to exist between sucrose and inulin with regard to an increased specificity of the enzyme towards that substrate present in a higher concentration.

Negoro and Kito (1973^{a}) screened various strains of yeasts for their ability to synthesize inulase and demonstrated that Candida kefyr possessed a β-fructofuranosidase with a high degree of hydrolytic activity towards inulin. C. kefyr was also found capable of synthesizing both an intracellular and extracellular inulase when cultured in a medium containing lactose. Purified preparations of both enzymes revealed a high hydrolytic activity towards inulin, sucrose and raffinose but no enzyme activity with respect to melezitose (Negoro, 1973). Negoro and Kito (1973^{b}) investigated the rate and completeness of inulin hydrolysis using an intracellular inulase isolated from C. kefyr and reported that inulin concentrations of 1% and 15%, were shown to be 95% and 85% hydrolyzed within 3 hours, respectively; nearly 100% hydrolysis of inulin was reported at both concentration levels after 24 hours. Negoro (1973) reported

that both the intracellular and extracellular inulase preparations followed a similar pattern during the time course of inulin hydrolysis. It was thereby proposed that the hydrolytic reactions of both enzymes took place via similar mechanisms. Ratios for the rates of sucrose and inulin hydrolysis for both enzymes were investigated by Negoro and Kito (1973[b]) and Negoro (1973) who reported that the S/I ratios for purified intracellular and extracellular inulases were 41.7 and 46.6, respectively; the S/I ratio for the extracellular inulase preparation remained unaltered during the course of purification and maximum inulase activity was observed at pH 4.5 for both the intracellular and extracellular inulases. Inulase pH stability studies determined that both extracellular and intracellular inulases were shown to remain relatively stable within a pH range of 4 - 8 (Negoro, 1973). It was reported, however, that the extracellular inulase was more labile towards pH levels greater than or less than 8.0 and 3.0, respectively, when compared to the intracellular inulase. Intracellular inulase showed a rapid loss in enzyme stability at pH levels less than 2.0 and greater than 8.5 (Negoro and Kito, 1973[b]). Negoro (1973) found that the optimum temperature required for maximum inulase activity for both enzymes was 50°C. The temperature activity curve for the extracellular enzyme was very similar to that of the intracellular enzyme activity curve (Negoro and Kito, 1973[b]; Negoro, 1973). Purified inulase prepared in pH 4.8 acetate buffer, heated for 10 minutes at temperatures ranging from 40 - 70°C was monitored for residual activity at pH 4.5

employing a temperature of 40°C. The temperature stability patterns for both enzymes remained relatively stable below 55°C but decreased at temperatures above 60°C (Negoro, 1973). Complete inactivation of intracellular inulase occured at 65°C within 10 minutes (Negoro and Kito, 1973[b]). During partial inactivation (i.e. time and temperature) of the inulase, the S/I ratio remained fairly constant.

Negoro and Kito (1973[b]) and Negoro (1973) examined the inhibitory effects of urea, phenylhydrazine, p-chloromercuribenzoate, mercuric chloride and silver nitrate on inulase activity. Increasing levels of urea were noted to correspond to a higher degree of inulase inactivation with a 4 M solution completely destroying all enzyme activity. The extracellular inulase was shown to display a greater tolerance towards inhibition at similar urea concentrations as compared to the intracellular inulase. Enzyme assays with inulase preparations incubated with 2 M urea for one hour resulted in residual enzyme activities of 50% for extracellular inulase and 20% for the intracellular inulase. Inhibitory action of 5×10^{-3} M phenylhydrazine displayed 23% more inhibition towards intracellular residual activity compared with extracellular activity. The inactivation of intracellular inulase was shown to be approximately twice as susceptible to (2×10^{-5} M) p-chloromercuribenzoate as compared to extracellular inulase. The inhibitory effect of p-chloromercuribenzoate and phenylhydrazine on inulase activity was shown to be more detrimental towards inulin hydrolysis as opposed to sucrose hydrolysis (Negoro and Kito, 1973[b]). The inhibitory differences towards

the substrates sucrose and inulin was proposed to arise from steric hinderance since inhibition was more predominant with a larger molecular weight molecule (inulin) as opposed to the smaller sucrose molecule. Studies using silver nitrate and mercuric chloride at concentrations of 1×10^{-5} M and 1×10^{-4} M respectively, had similar inhibitory effects towards inulase with the intracellular enzyme preparation retaining 50% more activity than the extracellular enzyme. Conformational differences between the two enzymes were considered a possibility in relation to the different inhibitory activities toward various inhibitors (Negoro, 1973).

FERMENTATION PRODUCTION OF INULASE

Bacteria and Molds

Controlled bacterial fermentation studies investigating the feasibility of inulase production have not been extensive. One of the main reasons for this resides in the inability of most bacterial strains to produce inulase. Several bacterial species, however, which have been investigated include Lactobacillus plantarum (Takahashi and Soutome, 1975[a]), Arthrobacter ureafaciens (Tanaka et al., 1972) and Erwinia phytophthorum (Tolba and Ghanem, 1959). Synthesis of an extracellular inulase by A. ureafaciens occured after 8 days in a chemically defined medium containing 1% inulin at 30°C. The initial pH of the liquid culture medium was adjusted to 7.0. Inulase production by E. phytophthorum using 2% inulin was noted after a cultivation period of 24 hours at 25°C. Inulase production utilizing various mold species has been reported by Kim (1975), Nakamura et al. (1978[a]) and Nakamura and Hoashi (1969). The studies reported were performed with the molds Aspergillus niger and Penicillium sp. The presence of inulin as part of the substrate was shown to be favourable for inulase production. Nakamura et al. (1978[a]) employed a 3% concentration of inulin during fermentation studies with A. niger; Kim (1975) and Nakamura and Hoashi (1969) on the other hand, utilized a crude inulin source (1 - 4%) obtained from Jerusalem artichoke with Penicillium. Other carbon sources investigated such as lactose, glucose and xylose were shown not to stimulate inulase production. Corn steep liquor was shown to promote inulase production by Penicillium. The

addition of corn steep liquor to the fermentation medium was also reported to serve as a suitable nitrogen source (Nakamura and Hoashi, 1969). Inulase production by Penicillium was shown to coincide with the utilization of sugars in the fermentation medium with maximal inulase yields occuring after 168 hours of fermentation (Nakamura and Hoashi, 1969). Kim (1975) indicated that Penicillium grown under optimal conditions showed a maximum inulase activity at 72 hours while optimal levels of extracellular inulase reported for A. niger occured after a fermentation period of 192 hours (Nakamura et al., 1978[a]).

The effect of temperature on inulase production by A. niger and Penicillium indicated that 30°C was optimal for both microorganisms; maximum extracellular inulase production by A. niger and Penicillium were obtained when the fermentation medium was initially adjusted to a pH of 4.5 (Nakamura et al., 1978[a]) and 5.0, respectively (Kim, 1975; Nakamura and Hoashi, 1969). Although the effect of oxygen tension was not reported, dynamic flask fermentation studies indicated certain requirements for oxygen in order to obtain maximum yields of inulase. Kim (1975) reported that static cultures examined for inulase production attained enzyme levels which were significantly lower than similar cultures grown under dynamic conditions.

Yeasts

The yeasts represent the major organisms in which inulase production have been studied. Those yeasts which have been investigated include: Saccharomyces fragilis (Kluyvermyces fragilis) by Snyder and Phaff (1960), Saccharomyces lactis by Yurkevich and Kovaleva (1972[a]),

Debaromyces cantarellii by Beluche et al. (1980), Candida salmenticensis by Guiraud et al. (1980) and Candida kefyr by Negoro and Kito (1973[a]). In addition, studies have been performed with various strains — S. fragilis, S. fragilis strain No. 351 (Nahm and Byun, 1977; Byun and Nahm, 1978; Snyder and Phaff, 1960; 1962), S. fragilis ATCC 12424 (GrootWassink and Fleming, 1980) and S. fragilis strain No. 0541 by Negoro (1978).

The fermentation media used for inulase production normally contained inulin concentration levels from 0.5% - 2.0% (Beluche et al., 1980; Negoro, 1978; Snyder and Phaff, 1962). Nahm and Byun (1977) obtained an extracellular inulase source from S. fragilis using a medium containing 10% j. artichoke tuber extract. These authors also reported that higher inulase yields could be attained by using tuber extracts as a carbon source as opposed to standard inulin. The maximum yield of extracellular inulase reported by Byun and Nahm (1978) was 0.5 units per ml of culture broth after 36 hours of fermentation. Snyder and Phaff (1960) obtained 0.3 units per ml of extracellular inulase activity from S. fragilis cultured in an inulin-containing medium for 48 hours. One unit of inulase activity was expressed as that amount of enzyme which releases 1 µ mole of fructose per minute at 30°C and pH 5.0. GrootWassink and Fleming (1980), however, reported that inulase production by S. fragilis was lowered by as much as 35% when j. artichoke fructans were used. The lower enzyme yields obtained with tuber extracts were postulated to be a consequence of carbon

catabolite repression. Inulase production using a continuous culture method employing S. fragilis was rapidly lowered by increasing the dilution rates thereby substantiating the sensitivity of inulase synthesis to catabolite repression (GrootWassink and Fleming, 1980). Continuous fermentation studies employing S. fragilis in a medium consisting of 1.0% sucrose and 0.5% yeast extract resulted in a two-fold increase in the total inulase activity per unit biomass as compared to batch fermentation studies. It was also shown that the percentage of recoverable inulase which was associated with the cells accounted for 50% and 70 - 75% of the total inulase produced in continuous and batch fermentations, respectively.

Time course production of inulase by S. fragilis and S. lactis employed an incubation temperature of 30°C (Byun and Nahm, 1978; Yurkevich and Kovaleva, 1972[a]). The yeasts, D. cantarellii and C. salmenticensis were reportedly grown at 28°C for optimum inulase production (Beluche et al., 1980; Guiraud et al., 1980). A study reported by Negoro (1978) indicated that maximum intracellular inulase activity for S. fragilis was found to have occured at 72 hours with a yield of 640 units/g of cells. A unit of inulase activity being the amount of enzyme which hydrolyzed 1 μ mole of reducing sugar per minute at 40°C at pH 4.5. Intracellular enzyme production initially paralleled the cell growth and eventually declined; cellular autolysis during the initial stages of the death phase was observed. Extracellular inulase activity in the culture medium was initially detected at lower levels during the exponential phase but rapidly

increased during the death phase. Guiraud et al. (1980) and GrootWassink and Fleming (1980) reported that for C. salmenticensis and S. fragilis, respectively, the maximum inulase activity prevailed during the stationary phase of growth. The stationary phase for S. fragilis was reached at 17 hours at which time the total inulase activity (intracellular plus extracellular) was 0.04 units (μg hexose/min/ml). Cultural fermentation of C. salmenticensis produced an optimum inulase yield of 312 and 54 (μ mole fructose/min for 100 ml of culture) for extracellular inulase and disrupted cells, respectively. Maximum intracellular inulase activities from S. lactis and D. cantarellii were noted at the termination of the fermentation — 18 and 48 hours, respectively (Beluche et al., 1980; Yurkevich and Kovaleva, 1972[a]). A specific inulase activity of 25 was reported for intact D. cantarellii cells, the specific activity being expressed as the μ mole fructose per min. per g cell protein (Beluche et al., 1980). Homogenized D. cantarellii cells were found to possess a higher specific activity of 55 μ mole fructose per min. per g cell protein. GrootWassink and Fleming (1980) using an autolytic process, demonstrated that cell disruption was required to fully expose all enzyme activity to the substrate inulin. A homogenized cellular preparation of S. lactis obtained by Yurkevich and Kovaleva (1972[a]) showed a specific activity of 3.4 units/mg protein. A unit of enzyme activity was described as the amount of enzyme required to hydrolyze 1 μ mole of sucrose per minute using 0.3 M acetate buffer at pH 3.3 and at 37°C. The initial pH levels of the culture

medium reportedly used for yeast fermentations ranged from 4.2 - 5.0 (Byun and Nahm, 1978; GrootWassink and Fleming, 1980; Negoro, 1978). Variable pH levels in the fermentation medium (3.5 - 6.0) had no consequence on the total inulase production by S. fragilis (GrootWassink and Fleming, 1980). It was noted, however, that fermentations adjusted to an initial pH of 6.0 produced a slightly higher biomass yield as opposed to pH 4.0. The effect of dissolved oxygen tension (DOT) on inulase production indicated that inulase synthesis occured below a DOT level of 2.5% (GrootWassink and Fleming, 1980). Furthermore, it was reported that a DOT ranging from 2.5% to 20.0% of saturation had no effect on biomass and inulase yields produced by S. fragilis. Snyder and Phaff (1960) obtained higher inulase yields with S. fragilis using aerated cultures as opposed to static cultures. The higher inulase levels were, however, attributed to increased biomass levels attained during the aerated fermentation rather than the inulase production being stimulated, per se.

The ability of C. kefyr to synthesize both an intracellular and extracellular inulase was investigated by Negoro and Kito (1973[a,b]) and Negoro (1973). These researchers found that the ratio of intracellular to extracellular inulase synthesized was influenced by the cultural conditions employed, specifically the composition of the medium and that significantly higher yields of inulase were produced in media containing 5% lactose as the carbon source as compared to similar carbon source concentrations of glucose, fructose or sucrose (Negoro and

Kito, 1973[a]). It was also postulated that the synthesis of both enzymes could possibly occur by different mechanisms. In addition, no extracellular inulase was produced when glucose served as the carbon source. The addition of either inulin or yeast extract to the culture medium was also found to produce a stimulatory effect on inulase production (Negoro and Kito, 1973[a]). Supplementation of 2% inulin into the basal medium yielded an increase in the intracellular and extracellular inulase activity of approximately 2.3 and 1.2 times, respectively. It was also demonstrated that total inulase production by C. kefyr was increased approximately 7 times upon addition of 0.35% yeast extract to the culture media. The effect of yeast extract on increased inulase yields was thought to be due to the vitamin content of the extract.

Optimum inulase production by C. kefyr was favoured by incubation temperatures ranging from 27 to 30°C, the latter temperature providing the highest inulase activity (Negoro and Kito, 1973[a]). Temperatures above 32°C drastically lowered the total inulase yield produced during the fermentation. Time course production studies of inulase reported by Negoro and Kito (1973[a]) indicated that intracellular inulase production paralleled cellular growth. Maximum intracellular inulase activity was reached after 72 hours of fermentation and was shown to decrease when cellular autolysis became apparent. It was noted that cellular autolysis occured after 64 hours of fermentation due to substrate exhaustion; the further addition of 1% inulin at this time was found to stimulate both intra and extracellular inulase production

but had no effect on biomass yield. The first appearance of extracellular inulase in the medium normally occured during the early exponential phase, however, maximum yields of the extracellular inulase activity were found to accumulate during the maximum exponential-initial stationary phase of the fermentation and continued to increase into the death phase. The extracellular inulase enzyme was thought to be distinct from the intracellular enzyme since it was released into the medium before cellular autolysis during the exponential growth phase. Differences in the enzymatic properties of the two isolated enzymes did, however, establish that C. kefyr does synthesize both an extracellular and an intracellular enzyme (Negoro and Kito, 1973[a]; Negoro, 1973). The culture media for C. kefyr was adjusted to pH 4.5 for inulase production studies.

MATERIALS AND METHODS

Microorganism

The yeast, Candida kefyr Strain No. 0616 was obtained from the Institute for Fermentation, Osaka, Japan.

Methods

1. Growth Medium

(a) Starch lactose inulin (SLI) medium.

SLI basal medium was used for inoculum preparation and flask fermentation studies. The medium composition as described by Negoro (1973[a]) consisted of the following:

Soluble Starch	0.5%
Lactose	5.0%
Inulin (dahlia tuber)	2.0%
Yeast Extract	0.35%
Urea	0.2%
KH_2PO_4	1.0×10^{-2} M
Distilled Water	250 ml

and was adjusted to pH 4.5 with 1 N HCL prior to sterilization. Monobasic potassium phosphate (KH_2PO_4) was autoclaved separately. The urea stock solution was sterilized by membrane filtration, using a 0.22 µm filter (Millipore Corporation, Bedford, Mass.).

2. Maintenance and Cultivation of Microorganism

Stock cultures were initially grown at 30°C for 48 hours on 3.9% Potato Dextrose Agar (PDA) slants adjusted to pH 3.5. The slants were held at 4°C and transferred monthly.

Unless otherwise stated, all flask fermentations were performed using SLI medium incubated at 10°C on a New Brunswick gyratory shaker, at 150 rev min^{-1}. The fermentations were terminated at 168 hours. During this time samples were asceptically withdrawn (25 ml aliquots) every 24 hour period.

The pH of the culture broth was monitored throughout the fermentation using a Fisher Accumet Model 520 digital pH/ion meter.

3. Dry Weight

Culture samples (25 ml) were suction filtered through cellulose acetate membrane filters with 0.45 μm pore diameters. The resultant yeast mats were each washed with approximately 20 ml sterile saline solution then placed with the filter membranes in vials, frozen at -20°C, freeze-dried and weighed to 0.1 mg accuracy. All vials were stored at -20°C until assayed.

The freeze-dried yeast mats were analyzed for intracellular inulase while the filtered broths were analyzed for extracellular inulase activity.

4. Preparation of Pre-inocula

Vegetative cells grown on PDA slants were inoculated into 200 ml sterile SLI medium and incubated at 30°C using a gyratory shaker operating at 150 rev min^{-1}. The resultant growth was harvested after 48 hours and asceptically centrifuged at 1,500 x g at 4 - 5°C for 10 minutes using a Sorval super-speed RC2-B centrifuge. The resultant supernatant was discarded. The yeast cake was asceptically washed 4 times with 0.85% sterile saline solution and similarly re-centrifuged. The final yeast cake was resuspended in 0.85% sterile saline solution and was used as the inocula for the flask studies. Inocula size ranged from 1.6 to 2.0 mg/ml fermentation broth.

5. Fermentor Cultivation

A Virtis, 4-litre bench-top fermentor, equipped with a magnetic drive impellor was used. The temperature of

fermentation was controlled by immersing the fermentor in a thermostatically controlled water bath.

Filtered air was fed into the fermentation vessel by means of a single probe sparger. Air was filter sterilized by means of glasswool, activated carbon air-line filters. Aeration was controlled by use of a flow-meter connected to an air-line pressure gauge. An aeration rate of 1.5 ml/min/ml medium was used. Fermentation studies were undertaken in duplicate.

6. Inulase Activity

Inulase activity was measured by monitoring the release of total reducing sugars from inulin as determined by the Shaffer-Somogyi Method. The enzyme assay consisted of incubating 3 ml of the respective enzyme preparation with 3 ml of 2% inulin dissolved in 2×10^{-2} M acetate buffer (pH 4.5) at $40^{\circ}C$ for 30 minutes. Intracellular inulase activity was determined by dissolving 6 mg of the lyophilized yeast in 3 ml of the buffer. The extracellular inulase consisted of 3 ml of filtered culture broth and 3 ml of the buffer. The enzymatic reaction was stopped after 30 minutes by boiling for 5 minutes. The amount of inulase which liberated 1 mg of reducing sugar after 30 minutes at $40^{\circ}C$ was defined as one unit of inulase activity.

Specific inulase activity was expressed as the units of enzyme per mg of protein. Total inulase is expressed as the combined specific activities of both intracellular and extracellular inulase.

7. Protein

The protein content was estimated by the Lowry Method (Lowry et al., 1951) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Growth and Time Course Production of Inulase

The growth and time course production of inulase by C. kefyr in SLI medium at 10°C is illustrated in Figure 2. The dry cell weight of the organism was shown to increase slowly up to 120 hours, thereafter an apparent stationary phase of growth was reached. The maximum dry cell weight, 4.5 mg/ml, was reached at 168 hours of fermentation. Intracellular inulase activity, during the course of growth, showed a sharp decrease for the first 48 hours followed by an increase which continued up to 168 hours, at which time the fermentation was terminated. The inulase activity achieved at this time was 0.60 units/mg. The extracellular inulase activity also showed an initial decrease which continued up to 96 hours. Thereafter, the extracellular inulase activity increased reaching a maximum (0.22 units/mg) at 144 hours. The maximum inulase yields produced by C. kefyr were shown to occur during the stationary growth phase, while the bulk of the enzyme synthesis was shown to occur during the short time interval towards the end of the active growth phase (declining growth phase).

Although similar patterns of enzyme production have also been observed by Hewitt (1981) working with K. fragilis using a batch fermentation, studies by Negoro and Kito (1973[a]) recorded that intracellular inulase production by C. kefyr at 30°C paralleled cellular growth. For most extracellular enzymes, production more or less parallels growth though

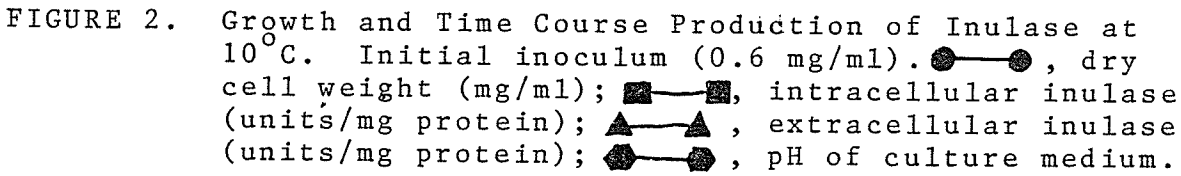
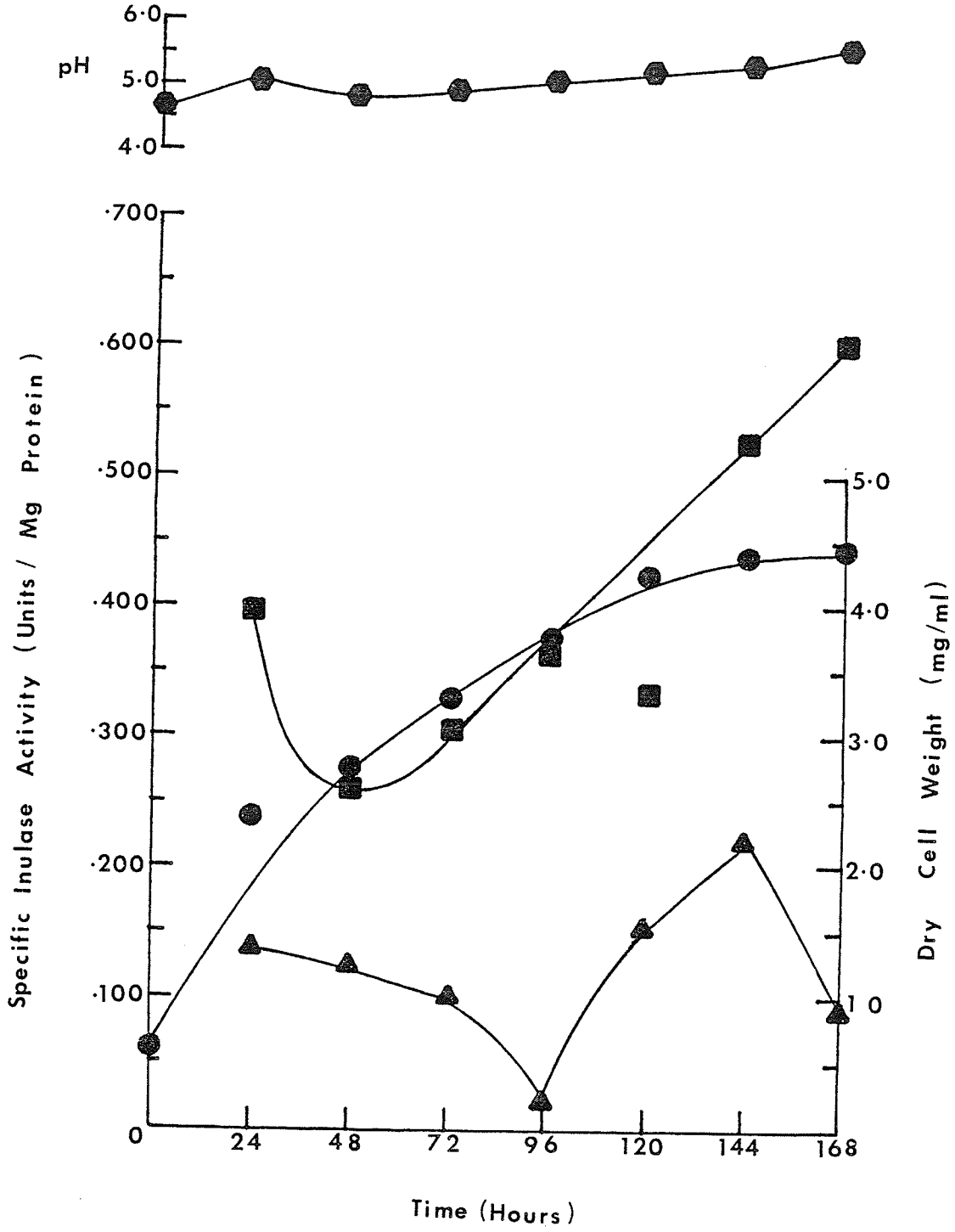


FIGURE 2. Growth and Time Course Production of Inulase at 10°C. Initial inoculum (0.6 mg/ml). ●—●, dry cell weight (mg/ml); ■—■, intracellular inulase (units/mg protein); ▲—▲, extracellular inulase (units/mg protein); ⬢—⬢, pH of culture medium.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Specific Inulase Activity (Units / Mg Protein)
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Dry Cell Weight (mg/ml)
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Time (Hours)

there may be small differences in the times at which different enzymes appear. Maximum enzyme yield is usually reached about the time that growth ceases (Davies, 1963). The intracellular inulase production in this study, however, did not coincide with the growth of the organism although maximum yields of inulase were noted at the declining growth phase. This lack of a growth associated increase in inulase could partially be attributed to the temperature ($10^{o}C$) which was employed in the study. Several researchers have cited different temperatures for optimal growth of the organism versus optimal temperature requirements for specific enzyme synthesis (Campbell, 1954; Mandels and Reese, 1960; Pan et al., 1950). GrootWassink and Fleming (1980) demonstrated that K. fragilis although optimally grown at 33 - $35^{o}C$, actively produced inulase at 26-$30^{o}C$. Since inulase production in Figure 1 continued until the fermentation was terminated, lack of inducers would otherwise halt enzyme synthesis. The initial dips in inulase production noted may be related to the carbon substrate concentration. Inulase synthesis by K. fragilis, for example, has been reported to be sensitive towards catabolite repression (GrootWassink and Fleming, 1980). Catabolite repression is a phenomenon whereby readily metabolized carbon substrates such as glucose, causes repression of enzyme synthesis (Magasanik, 1961). Several investigators have reported the appearance of inulase activity in growth media only when the simple carbon substrate concentration became limiting; that is, an inverse relationship between readily metabolizable sugars and enzyme synthesis was observed (Beluche et al., 1980;

Negoro and Kito, 1973[a]). In this study the pre-fermentation inoculum was grown at 30°C for 48 hours in SLI medium. Although residual sugars were not monitored, it is possible that at the time of their transfer, they contained a high level of inulase activity presumably due to the exhaustion of the more readily available carbohydrates, and induction by inulin. Negoro and Kito (1973[a]) using a similar growth medium reported that at 30°C, C. kefyr produced maximum inulase levels when the reducing sugar concentration became limiting. Also, inulase has reportedly been suggested to be of an inducible nature in the presence of inulin (Beluche et al., 1980; Guiraud et al., 1980; Snyder and Phaff, 1960). The combination of low levels of readily metabolizable sugars and availability of inulin in the pre-fermentation medium stimulating inulase induction, could result in inoculum cells having a high inulase activity, hence the higher inulase activity noted at the start of the fermentation. The decrease in inulase activity as shown in Figure 2 possibly depicts the repression of inulase synthesis as a consequence of the recently transferred inoculum utilizing readily available sugars in the fresh medium. It is also conceivable that the increase in inulase activity coincides with the depletion of readily available sugars initially present in the medium. Although residual sugar was not monitored during the course of fermentation, similar results have been obtained by Negoro and Kito (1973[a]) and Beluche et al. (1980) working with C. kefyr and D. cantarellii, respectively. Although the exact nature and location of the inulases produced by C. kefyr are not well characterized, the cellular inulase of

K. fragilis has been demonstrated to be primarily associated with the cell wall and is rapidly released during protoplast formation. This cellular or intracellular inulase is not physically bonded to the cell wall, but is prevented from being secreted by the intact cell wall which serves as a permeability barrier. Since sugars cannot penetrate the yeast plasma membrane, they must be carried across by specific transport mechanisms. Constitutive or inducible carriers exist for monosaccharides and some disaccharides but no such mechanism exists for inulin. Instead, inulin must be hydrolyzed extracellularly to monosaccharides which can then be transported. The secretion of extracellular inulase would then presumably occur once intracellular inulase was in place (induction and/or relaxation of catabolite repression). Extracellular inulase levels increased towards the end of exponential growth approximately 48 hours after initial increases were shown by intracellular inulase. Both C. kefyr and K. fragilis demonstrated a delayed release of an extracellular inulase into the fermentation medium when studied by Negoro and Kito (1973[a]) and Negoro (1978), respectively. The delayed release of extracellular inulase may be part of the cells attempt to further procure inulin as the medium becomes depleted of fermentable carbohydrates. At 144 hours, peak extracellular inulase activity was reached after which it decreased. This decrease may represent initial signs of inulin depletion and/or effects of stationary growth.

Since the production of inulase is apparently dependent,

among other factors, on the availability and concentration of readily metabolizable carbohydrates it was of interest to determine inulase production using a heavier inoculum. It was thought that a heavier inoculum would deplete available carbohydrates faster, lessening the length of catabolite repression and at the same time increase the yield of enzyme synthesized, since Snyder and Phaff (1960) reported that higher cell yields with aerated cultures were responsible for increased inulase levels rather than stimulation of the enzyme production.

The time course production of inulase using a higher inoculum level is illustrated in Figure 3. The results indicate that maximum biomass (6.2 mg/ml) was reached at 144 hours using an inoculum of 1.8 mg/ml at 0 hours. This is in contrast with a maximum biomass (4.5 mg/ml) reached at 144 hours using an inoculum of 0.6 mg/ml at 0 hours (Figure 2). Both growth curves showed similar biomass patterns approaching an apparent stationary phase at 120 hours. Inulase production levels were increased slightly when a higher initial inoculum rate was used as shown in Figure 3. The maximum activities achieved during the fermentation period were 0.77 units/mg at 168 hours (intracellular) and 0.29 units/mg at 96 hours (extracellular). The higher inulase levels obtained can probably be attributed to the increased cell yield, since no fermentation parameters were adjusted other than utilizing a heavier inoculum. Although increased yields of inulase were obtained, the results were not totally as expected. The use of a heavier inoculum would be expected to deplete

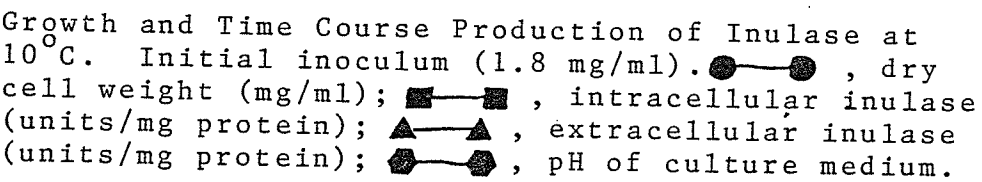
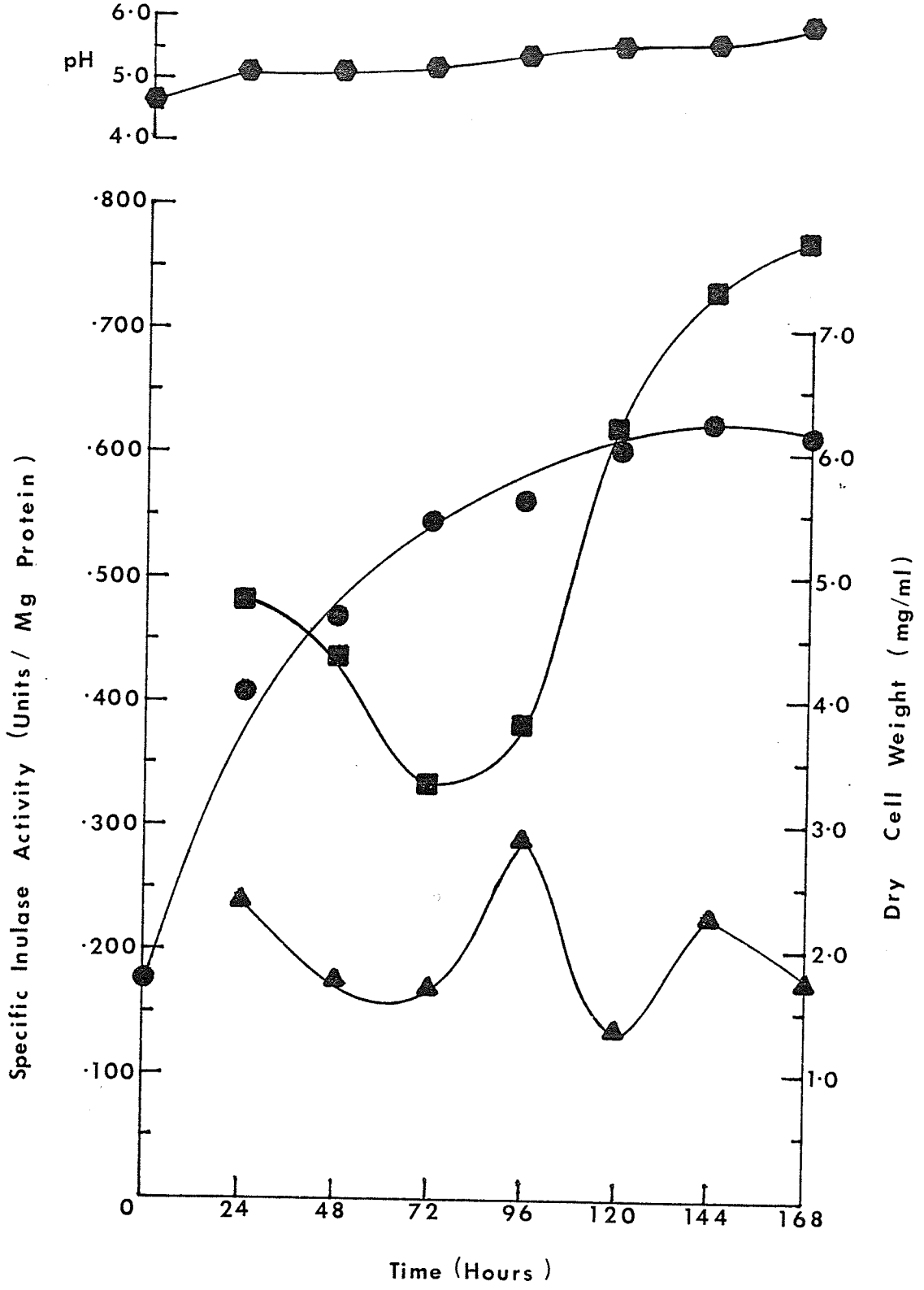


FIGURE 3. Growth and Time Course Production of Inulase at 10°C. Initial inoculum (1.8 mg/ml). , dry cell weight (mg/ml); , intracellular inulase (units/mg protein); , extracellular inulase (units/mg protein); , pH of culture medium.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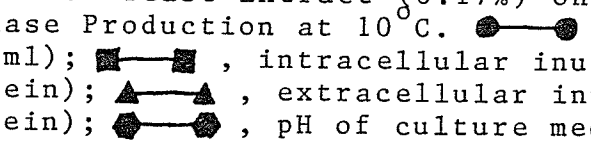
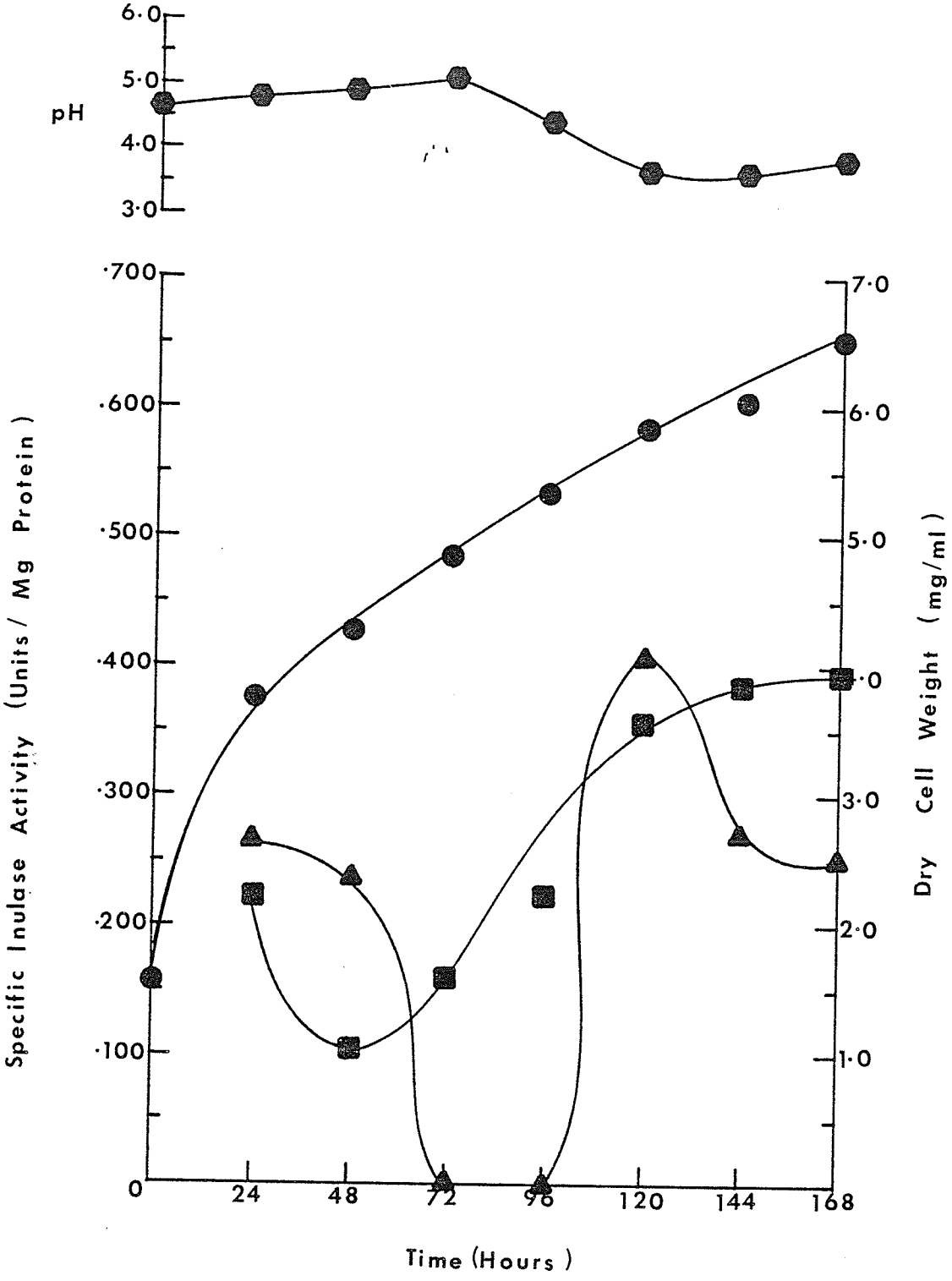
Specific Inulase Activity (Units / Mg Protein)
Dry Cell Weight (mg/ml)
Time (Hours)

available carbohydrates much more quickly and as such see an earlier rise in the intracellular inulase. This was not observed. Intracellular inulase increased only after 72 hours with the heavier inoculum while it increased 24 hours sooner with the lighter inoculum. Alternately, the delay in the rise of the intracellular inulase may be attributed to the cells secretion of an extracellular inulase into the medium at 72 to 96 hours, therby a delayed rise in intracellular level would be expected. Negoro and Kito (1973[a]) reported that C. kefyr grown at 30°C similarly secreted an extracellular inulase during the exponential phase of growth. The apparent delay in the production of intracellular inulase and the earlier rise of the extracellular inulase peak could then lead to a more rapid utilization of inulin, thereby lessening the need for intracellular inulase synthesis. Figure 3 illustrates what appears to be a rise in extracellular inulase activity after a time period of 144 hours. Negoro and Kito (1973[a]) stated that increased inulase levels within the medium may be partiallly related to the release of intracellular inulase during cell autolysis. This may also have been observed in the latter portion of the fermentation particularly during the death phase. The possible release of intracellular inulase could partially account for the relatively sharp rise in extracellular inulase activity at 144 hours.

The Effect of Yeast Extract on Growth and Inulase Production

The effects of yeast extract on inulase production using SLI medium are illustrated in Figures 4, 5 and 6. Although the growth pattern and biomass accumulation were very similar in each of the above fermentations, the time course production of intracellular and extracellular inulase were shown to vary. Maximum biomass accumulation at 168 hours differed only slightly — 6.1, 6.5 and 6.7 mg/ml using 0.17%, 0.35% and 0.53% yeast extract. Although yeast extract appeared to offer no beneficial effects as far as biomass accumulation, it was observed that the effects of yeast extract were much more pronounced on inulase activity. Intracellular inulase activity showed similar profiles of production although activities were shown to differ. Yeast extract (0.35%) showed the highest level of intracellular inulase activity (0.77 units/mg) followed by 0.40 units/mg and 0.30 units/mg for yeast extract concentrations of 0.17% and 0.53%, respectively. The effect of yeast extract on extracellular inulase was shown to be deleterious, particularly for the higher concentrations. The extremely low levels of extracellular inulase (Figure 6) after 120 hours of fermentation as contrasted to Figures 4 and 5 support this effect. Also, the proportion of extracellular to intracellular inulase activity using 0.53% yeast extract was found to be the lowest of all the fermentations (Table 2). It would appear then, that 0.35% yeast extract does promote maximal total inulase production

FIGURE 4. Effect of Yeast Extract (0.17%) on Growth and Inulase Production at 10°C.  , dry cell weight (mg/ml); , intracellular inulase (units/mg protein); , extracellular inulase (units/mg protein); , pH of culture medium.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Specific Inulase Activity (Units / Mg Protein)
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Dry Cell Weight (mg/ml)
24
48
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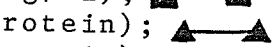
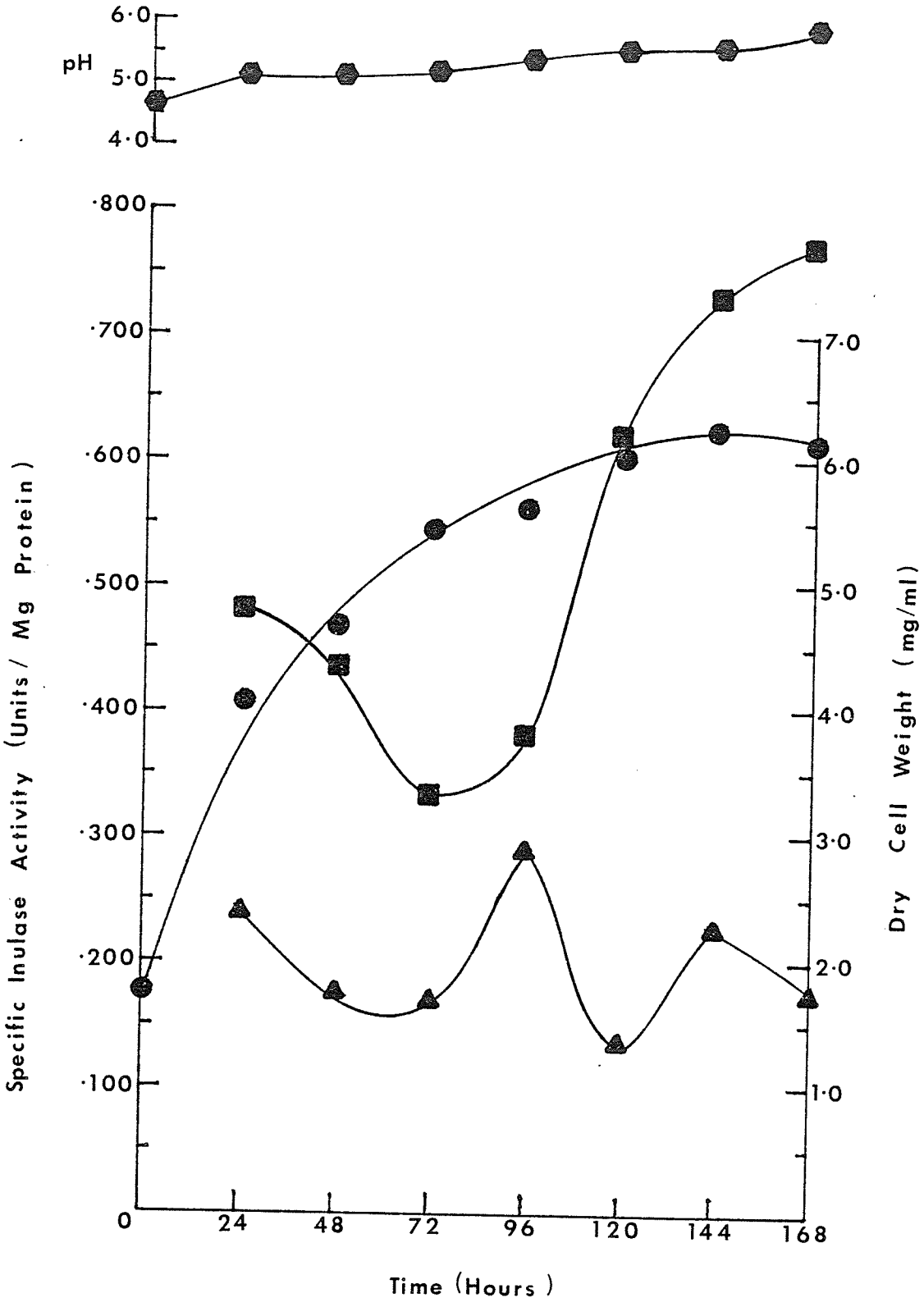
96
120
144
168
Time (Hours)

FIGURE 5. Effect of Yeast Extract (0.35%) on Growth and Inulase Production at 10°C.  , dry cell weight (mg/ml);  , intracellular inulase (units/mg protein);  , extracellular inulase (units/mg protein);  , pH of culture medium.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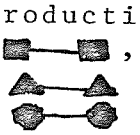
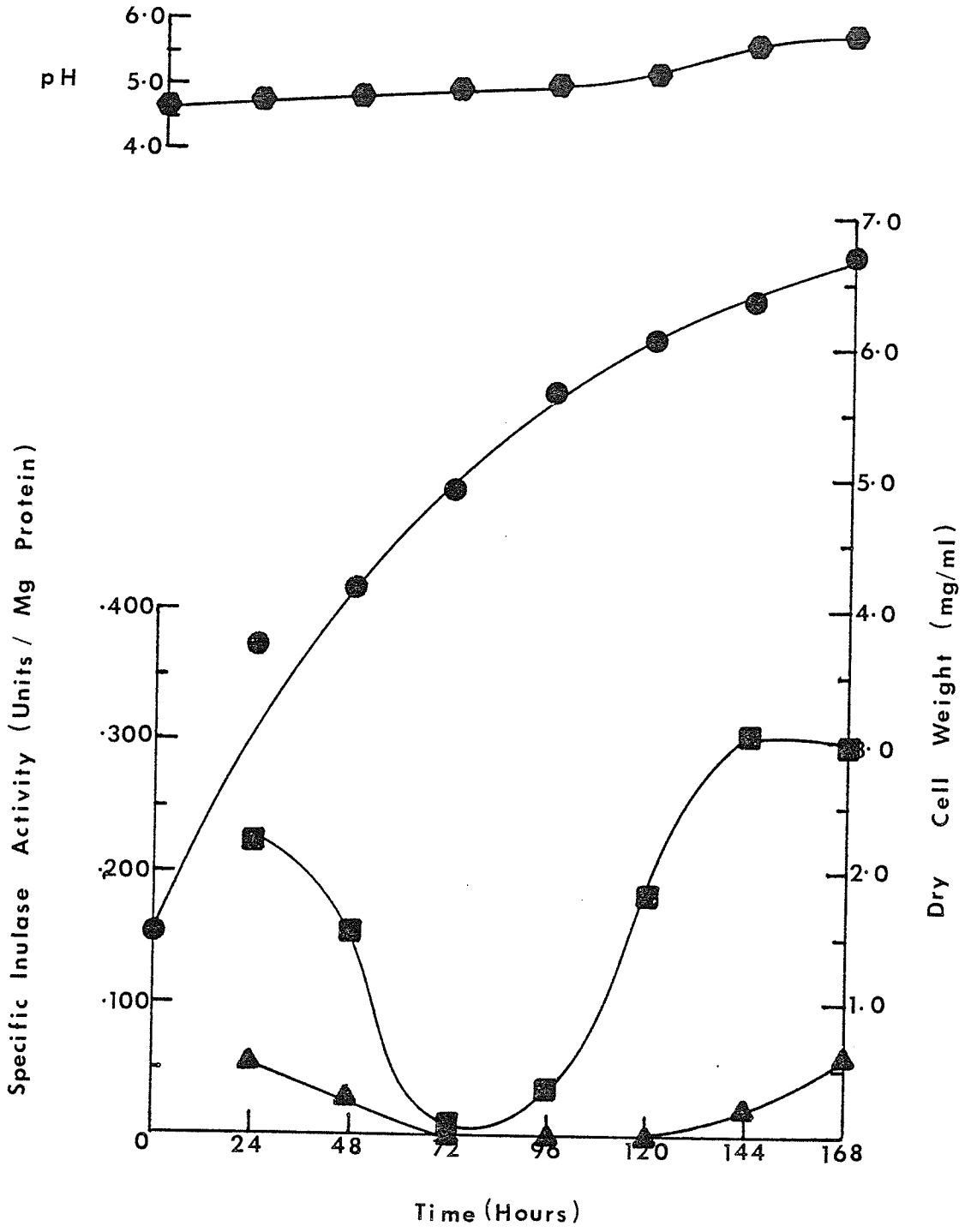
Specific Inulase Activity (Units / Mg Protein)
Dry Cell Weight (mg/ml)
Time (Hours)

FIGURE 6. Effect of Yeast Extract (0.53%) on Growth and Inulase Production at 10°C.  , dry cell weight (mg/ml); , intracellular inulase (units/mg protein); , extracellular inulase (units/mg protein); , pH of culture medium.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Specific Inulase Activity (Units / Mg Protein)
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Dry Cell Weight (mg/ml)
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1·0
24
48
72
96
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144
168
Time (Hours)

TABLE 2. Disposition of Intracellular and Extracellular Inulase Activity Using Varying Levels of Yeast Extract.

	0.17%			0.35%			0.53%		
Time (hrs)	Total[a] Inulase	% Extra	% Intra	Total[a] Inulase	% Extra	% Intra	Total[a] Inulase	% Extra	% Intra
24	0.484	54.1	45.9	0.716	33.0	67.0	0.277	19.8	80.2
48	0.340	69.7	30.3	0.608	28.4	71.6	0.182	15.9	84.1
72	0.160	00	100.0	0.499	33.3	66.7	0.005	00	100.0
96	0.223	00	100.0	0.670	42.8	57.2	0.035	00	100.0
120	0.765	53.1	46.9	0.751	17.7	82.3	0.183	00	100.0
144	0.653	40.9	59.1	0.955	23.6	76.4	0.324	6.2	93.8
168	0.645	38.8	61.2	0.941	18.2	81.8	0.356	16.8	83.2

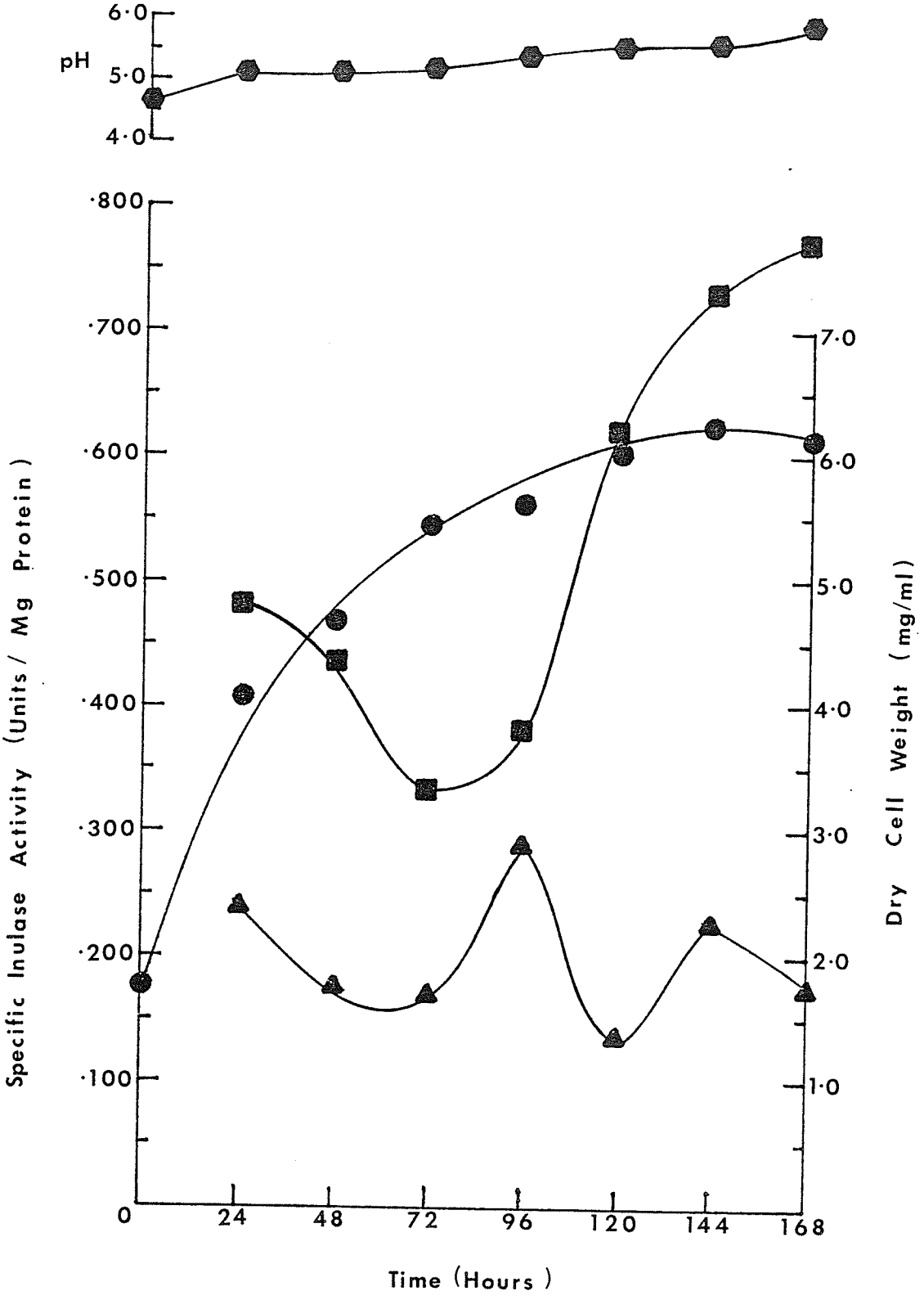
a Total inulase is expressed as specific inulase activity (units/mg protein). The total inulase is the combined intracellular and extracellular inulase activity.

and a higher yield in intracellular inulase activity. Yeast extract has been shown to be necessary and beneficial for many fermentations. Some of the components which have been identified in yeast extract are known to be required for growth. The usual response to complex nitrogen sources was increased enzyme synthesis although growth was not affected. Factors involved which may effect enzyme synthesis may be the result of the available amino acids and/or peptides present or due to high levels of growth factors and/or trace elements (Davies, 1963). Some heavy metals such as manganese, copper and iron contained in yeast extract are required in low concentrations for respiration and/or enzyme synthesis but at greater concentrations they can become toxic to the organism and/or enzyme synthesis (Suolmalainen and Oura, 1971). Davies (1963) reported lowered proteinase production by Bacillus subtilis when over 0.5% peptone was used in the medium. Vitamins contained within yeast extract may also effect cell growth and enzyme production (Koser, 1962). A review of literature has not provided any information as to the requirement of co-factors or activators which would explain the beneficial or detrimental effects on inulase production. Recent studies by Hewitt (1981) have indicated that yeast extract may cause transient repression dependent on its concentration in the growth medium.

Effect of Aeration on Inulase Production

The effect of aeration on inulase production by C. kefyr at 10°C is illustrated in Figures 7, 8 and 9. The dissolved oxygen tension (DOT) for these respective fermentations was determined to be greater than 20% of saturation. As shown in Figures 7 and 8, increasing the rate of gyratory agitation from 150 to 300 rev min^{-1} resulted in a subsequent increase both in the rate and maxima biomass accumulation. It was noted that a higher dry cell weight gain of 5 mg/ml as opposed to 3.6 mg/ml was obtained after 72 hours of fermentation utilizing 300 rev min^{-1}. Although the maximum levels of intracellular inulase obtained were similar (0.77 units/mg, 150 rev min^{-1}; 0.76 units/mg, 300 rev min^{-1}), the pattern of enzyme production was shown to differ. Fermentations (300 rev min^{-1}) showed a steady increase in intracellular inulase activity somewhat parallel to biomass accumulation. This effect was also demonstrated by Negoro and Kito (1973[a]) and Negoro (1978) using C. kefyr and K. fragilis, respectively. This time course production of intracellular inulase in Figure 8 is in sharp contrast to flask studies at 150 rev min^{-1} as shown in Figure 7. The decrease in the intracellular inulase activity (dip) noted in Figure 7 disappeared with increased agitation. Supposedly, the increased rate in biomass accumulation resulted in a faster utilization of readily available fermentable sugars, thereby lessening any inulase suppression effect. Extracellular inulase in both fermentations exhibited a somewhat similar pattern.

FIGURE 7. Effect of Agitation Rate (150 rev min^{-1}) on Growth and Inulase Production at 10°C. ●—● , dry cell weight (mg/ml); ■—■ , intracellular inulase (units/mg protein); ▲—▲ , extracellular inulase (units/mg protein); ⬢—⬢ , pH of culture medium.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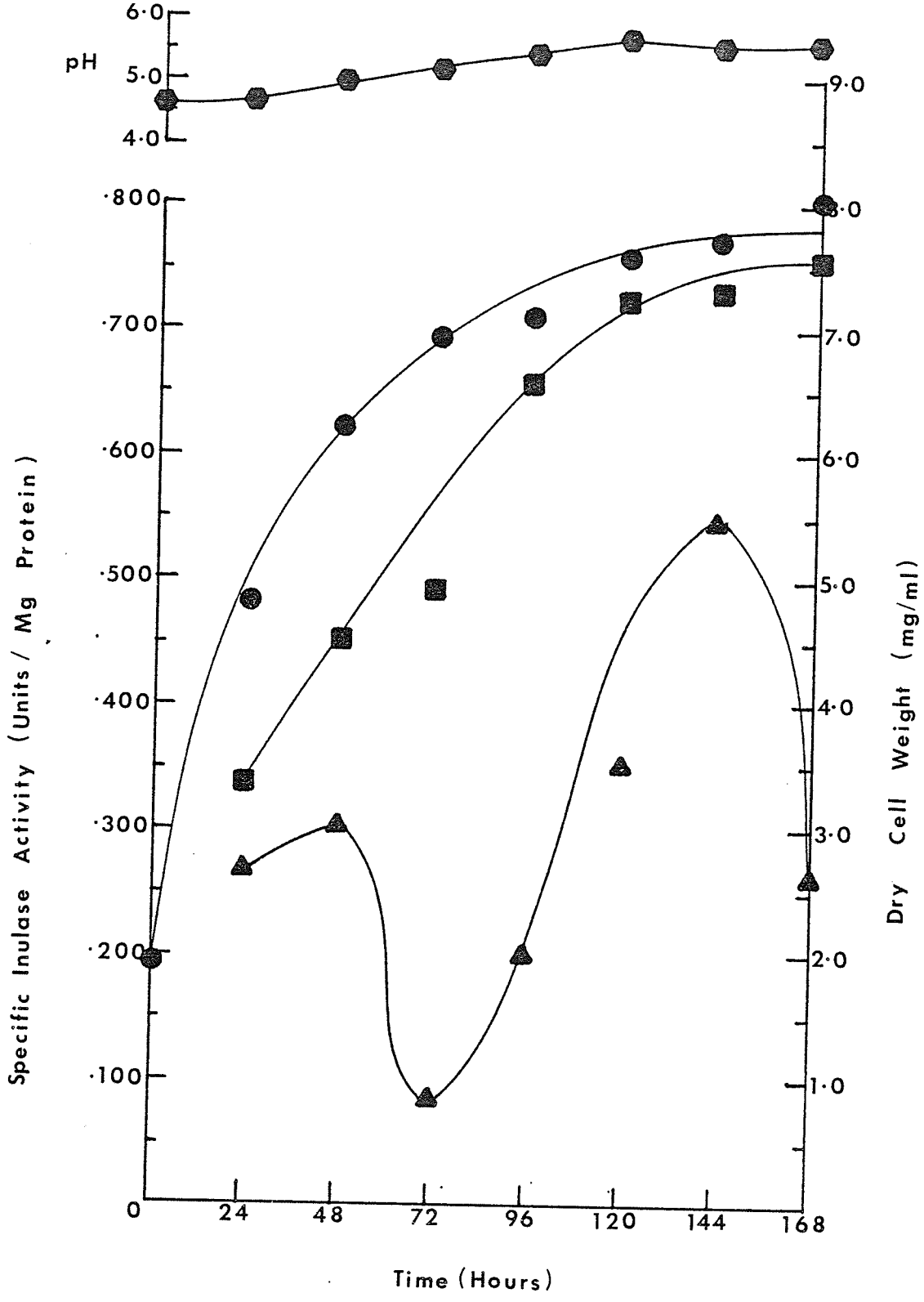
24
48
72
96
120
144
168
Specific Inulase Activity (Units / Mg Protein)
Dry Cell Weight (mg/ml)
Time (Hours)

FIGURE 8. Effect of Agitation Rate (300 rev min^{-1}) on Growth and Inulase Production at 10°C. ●—● , dry cell weight (mg/ml); ■—■ , intracellular inulase (units/mg protein); ▲—▲ , extracellular inulase (units/mg protein); ⬢—⬢ , pH of culture medium.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96
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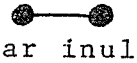
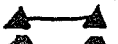
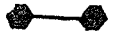
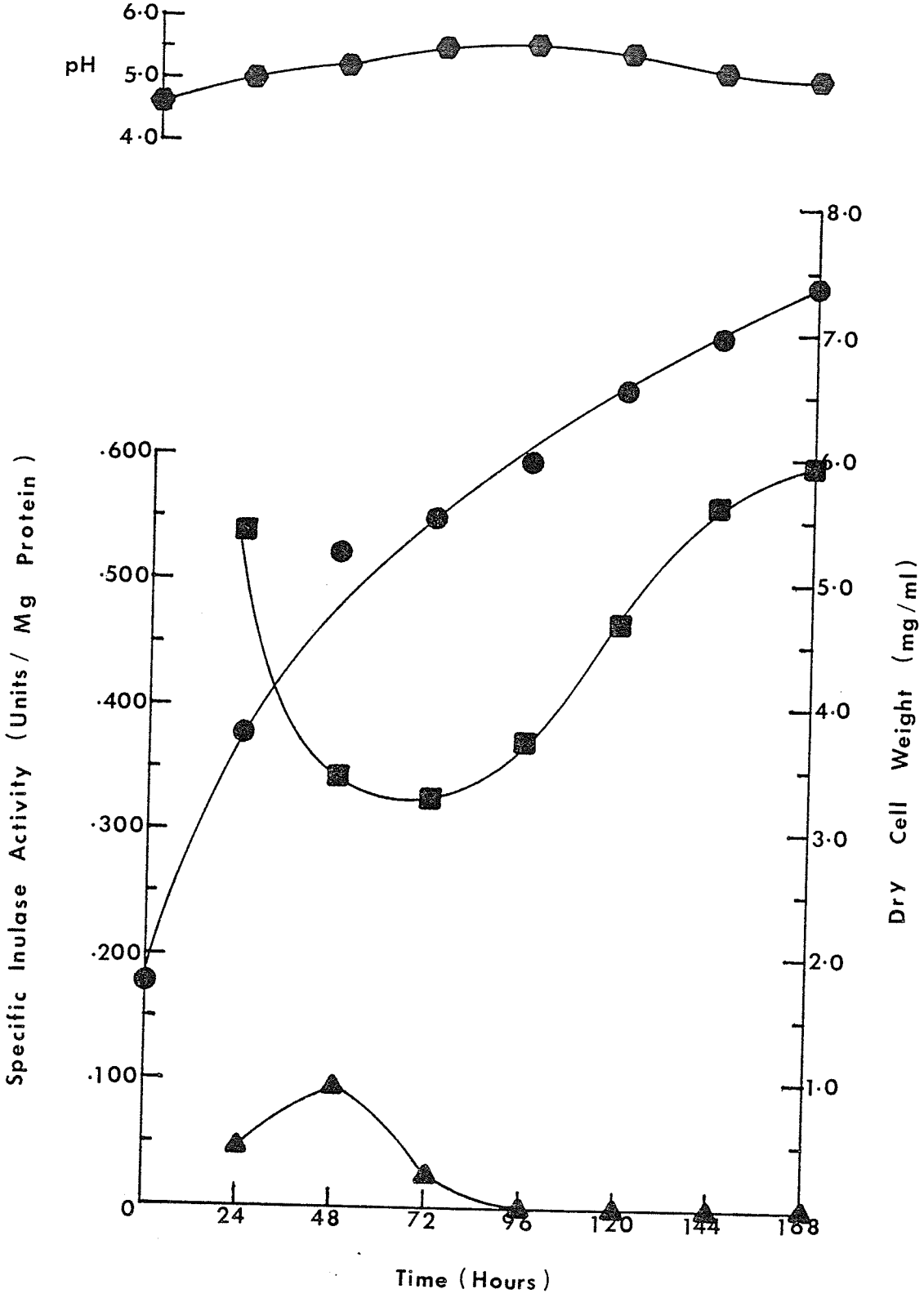
144
168
Specific Inulase Activity (Units / Mg Protein)
Dry Cell Weight (mg/ml)
Time (Hours)

FIGURE 9. Effect of Agitation Rate (500 rev min^{-1}) and Aeration (1.5 ml/min/ml fermentation liquid) on Growth and Inulase Production at 10°C.  , dry cell weight (mg/ml);  , intracellular inulase (units/mg protein);  , extracellular inulase (units/mg protein);  , pH of culture medium.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Specific Inulase Activity (Units / Mg Protein)
Dry Cell Weight (mg/ml)
Time (Hours)

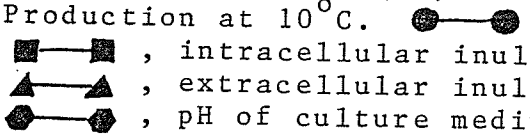
The increased growth rate and biomass accumulation (Figure 8) appears to have created a greater demand for a readily metabolizable carbon source. This resulted in an earlier secretion of extracellular inulase (24 to 48 hours) during the exponential phase. The apparent rise in extracellular inulase up to 144 hours could partially be attributed to the release of intracellular inulase during cellular and/or protoplast formation. At the higher agitiation rate (300), extracellular inulase was shown to decrease in activity from 48 - 72 hours. This decrease in activity may be attributed to the rapid utilization of other easily fermentable carbon sources. The subsequent increase in extracellular inulase activity would then be prompted by the rapid decrease in the concentration of hitherto easily available carbon sources. The final decrease in extracellular inulase activity is undoubtedly due to a lack of dynamic growth.

The effect of increased agitation supplemented with air infusion using a 7-litre fermentor on inulase production is illustrated in Figure 9. High agitation and/or aeration had a deleterious effect on the time course production of extracellular inulase. Suppression of intracellular inulase activity was also evident (Figure 9) when compared to the lower agitation rates illustrated in Figures 7 and 8. A similar decrease (dip) in the intracellular inulase activity became apparent during the early stages of the fermentation as depicted in Figures 2 and 3. As stated earlier carbon substrate concentration may be responsible for this dip. Extracellular enzyme production, showed a small maximum at 48 hours, after-which the activity decreased drastically. Some

extracellular enzymes are very susceptible to surface denaturation under conditions where the area of the gas - water interface is greatly increased, such as by vigorous gassing or shaking (Pappenheimer, 1955). Susceptibility to surface denaturation might be one factor involved where there appears to be an optimum aeration rate for enzyme formation. It has been documented that dynamic cultures have resulted in higher inulase activity as opposed to static cultures (Kim, 1975; Snyder and Phaff, 1960). In addition, the lack of a stationary phase would preclude any protoplast or lytic inulase showing up as extracellular inulase.

Effect of Tween 80 on Growth and Inulase Production

Reese and Maguire (1969; 1970) investigated the enzyme yields from fungal cultures and reported marked increases upon the addition of the surfactant Tween 80 (sorbitan polyoxyethylene monooleate. The effects of Tween 80 at concentrations of 0%, 0.5%, 1.0% and 1.5% w/v on inulase production by C. kefyr at 10°C are illustrated in Figures 10, 11, 12 and 13. The addition of Tween 80 to the fermentations gave rise to a gradual increase in biomass attained up to 1.0%. At levels of 1.5% there was no additional increase in biomass observed. It is possible that the effect of Tween 80 to the biomass could be more dramatic if the organism was to have been cultured at its optimum growth temperature utilizing inulin as a sole source of carbon. All levels of intracellular inulase activities tested throughout the time periods using Tween 80 were shown to be lower in comparison to the control fermentation. However, the same general pattern of intracellular enzyme production was observed for the fermentation containing Tween 80 as was shown for the fermentation in the absence of Tween 80. The pattern of extracellular activity showed marked differences from the control fermentation particularly in the levels of activity attained as shown in Figures 11 and 13. A rapid rise in extracellular activity was shown to occur from 24 to 48 hours reaching a maximum specific activity of 0.33 and 0.42 units/mg in the fermentation containing 0.5% and 1.5% concentrations of Tween 80, respectively. This increase in activity was followed by a decline in activity to 96 hours

FIGURE 10. Effect of Tween 80 (0%) on Growth and Inulase Production at 10°C.  , dry cell weight (mg/ml); , intracellular inulase (units/mg protein); , extracellular inulase (units/mg protein); , pH of culture medium.

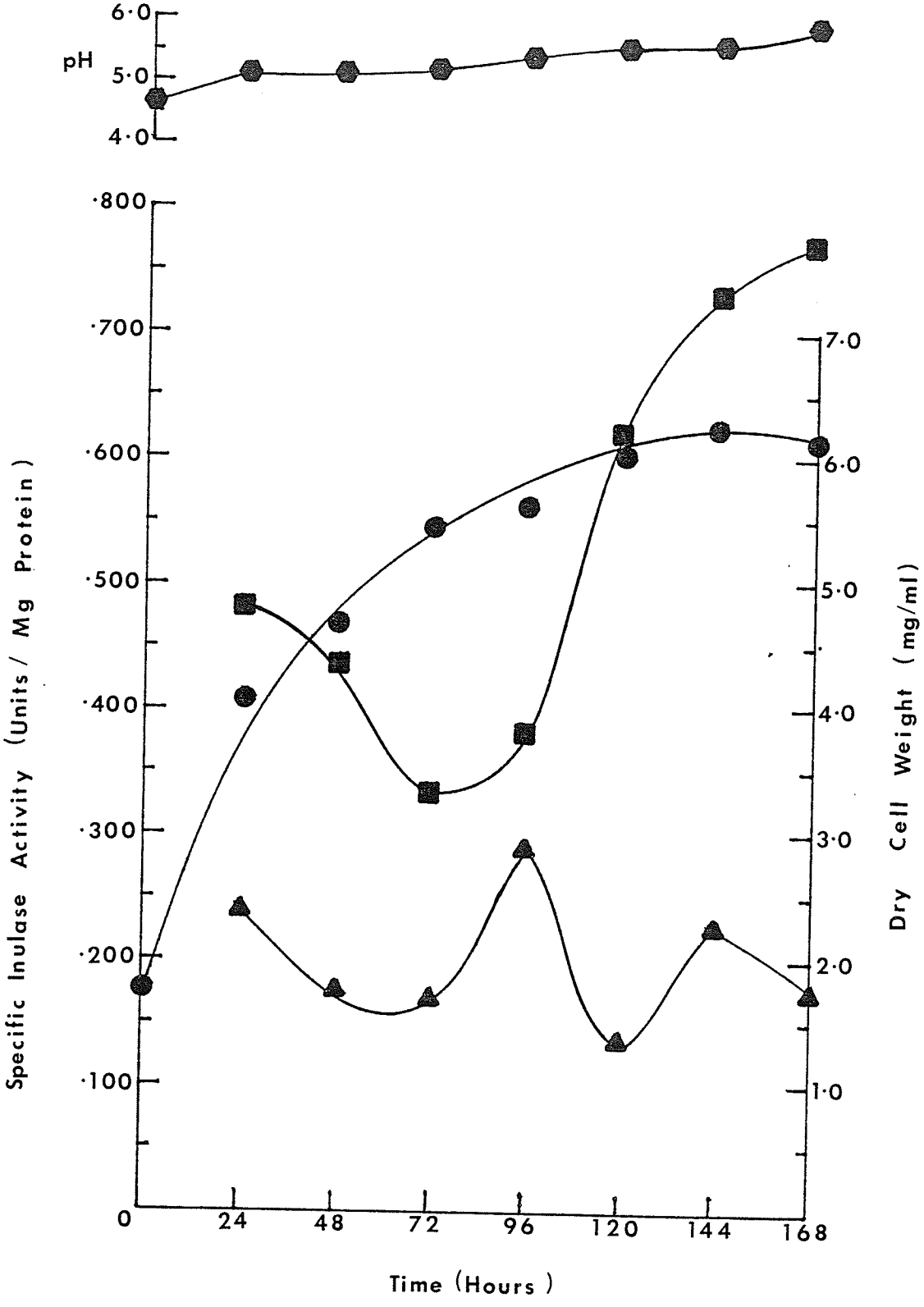
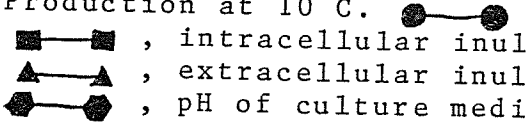

pH
6·0
5·0
4·0
·800
·700
·600
·500
·400
·300
·200
·100
0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
24
48
72
96
120
144
168
Specific Inulase Activity (Units / Mg Protein)
Dry Cell Weight (mg/ml)
Time (Hours)

FIGURE 11. Effect of Tween 80 (0.5%) on Growth and Inulase Production at 10°C.  , dry cell weight (mg/ml); , intracellular inulase (units/mg protein); , extracellular inulase (units/mg protein); , pH of culture medium.

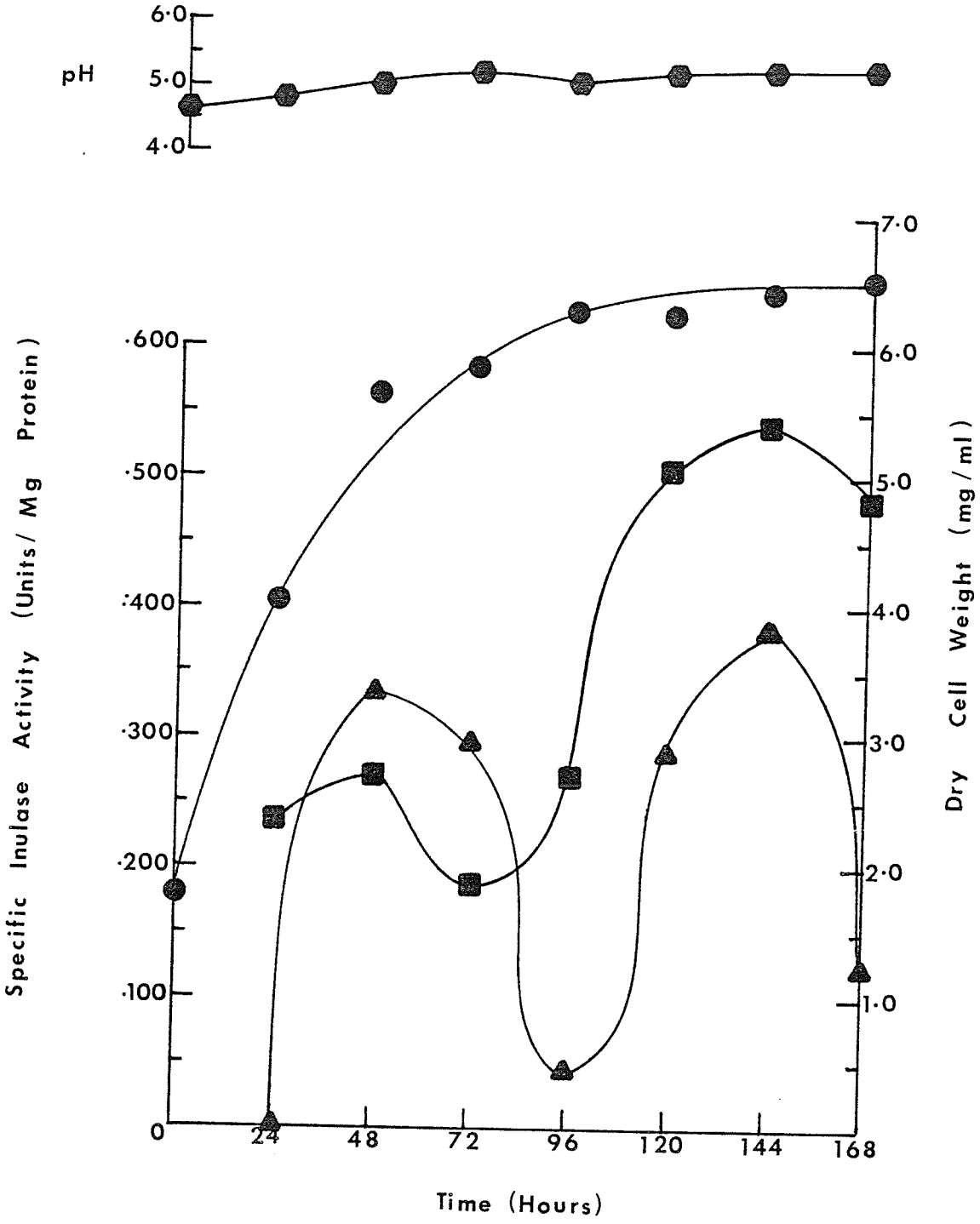
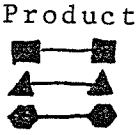

pH
6·0
5·0
4·0
Specific Inulase Activity (Units/ Mg Protein)
·600
·500
·400
·300
·200
·100
0
Dry Cell Weight (mg/ml)
7·0
6·0
5·0
4·0
3·0
2·0
1·0
24
48
72
96
120
144
168
Time (Hours)

FIGURE 12. Effect of Tween 80 (1.0%) on Growth and Inulase Production at 10°C.  , dry cell weight (mg/ml); , intracellular inulase (units/mg protein); , extracellular inulase (units/mg protein); , pH of culture medium.

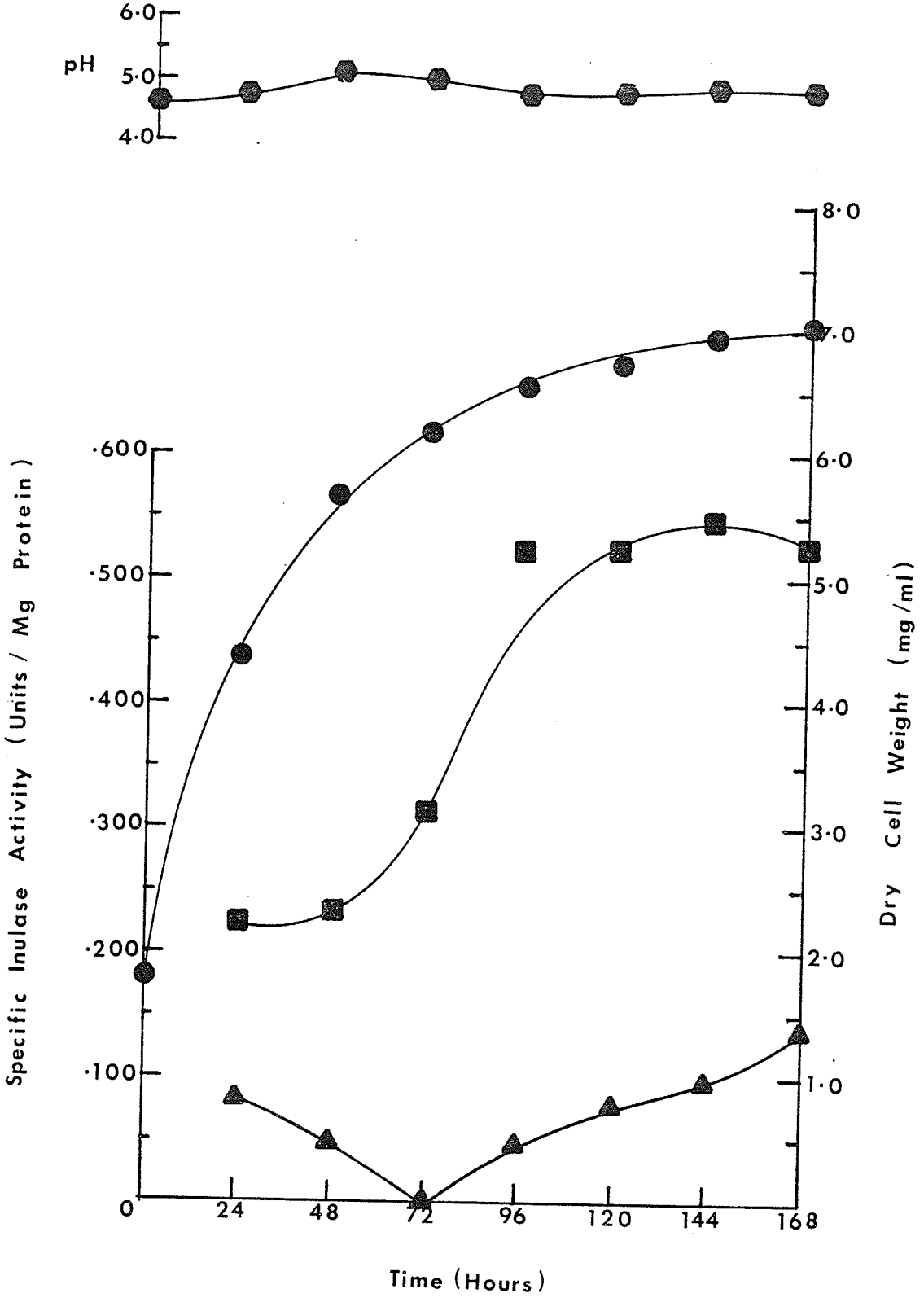
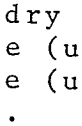
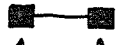
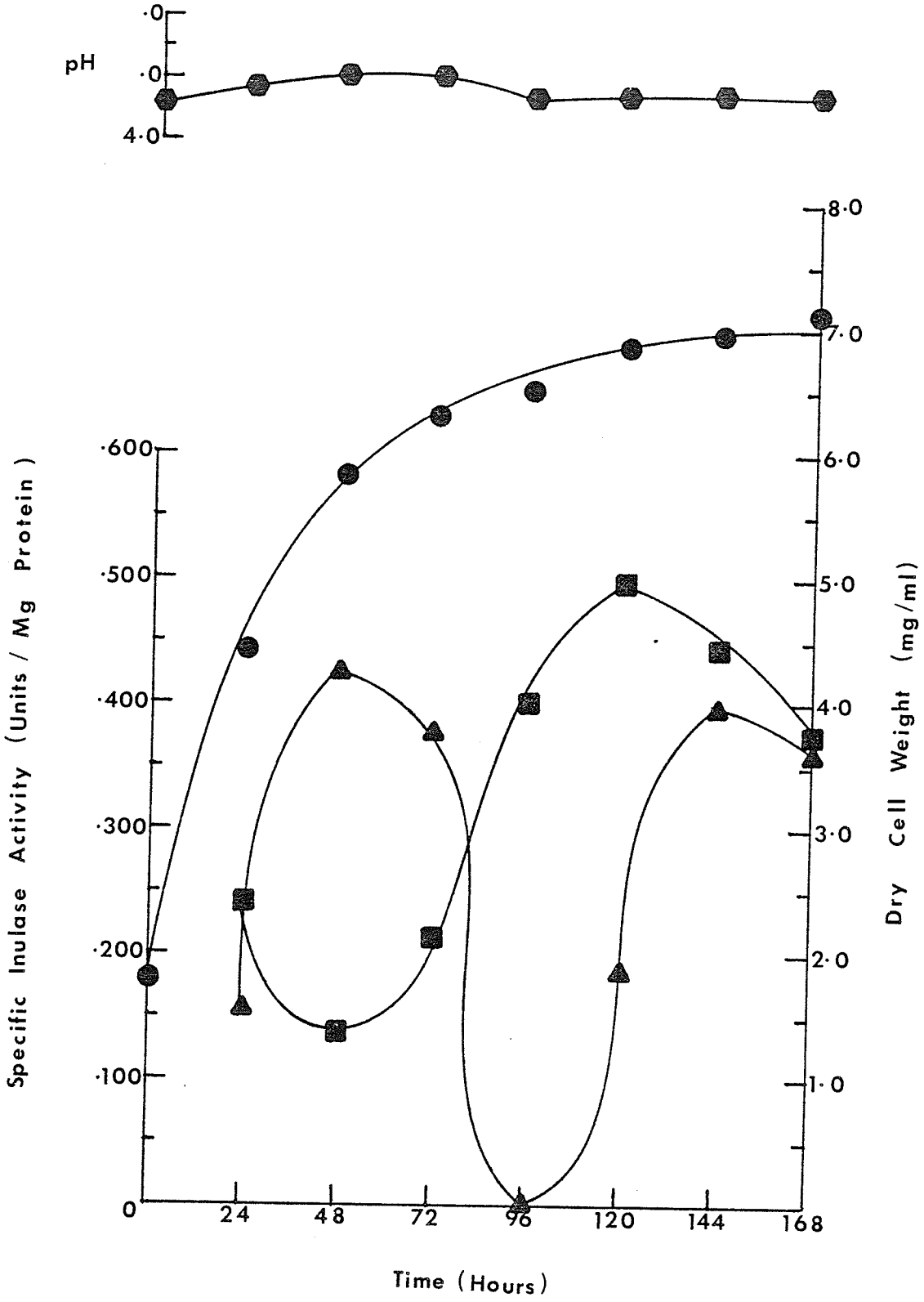

pH
6·0
5·0
4·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
·600
·500
·400
·300
·200
·100
0
24
48
72
96
120
144
168
Specific Inulase Activity (Units / Mg Protein)
Dry Cell Weight (mg/ml)
Time (Hours)

FIGURE 13. Effect of Tween 80 (1.5%) on Growth and Inulase Production at 10°C.  , dry cell weight (mg/ml);  , intracellular inulase (units/mg protein);  , extracellular inulase (units/mg protein);  , pH of culture medium.


pH
·0
·0
4·0
8·0
7·0
6·0
5·0
4·0
3·0
2·0
1·0
·600
·500
·400
·300
·200
·100
0
24
48
72
96
120
144
168
Specific Inulase Activity (Units / Mg Protein)
Dry Cell Weight (mg/ml)
Time (Hours)

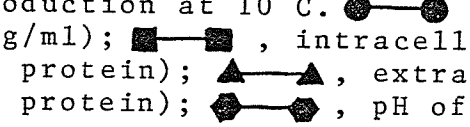
afterwhich a secondary rise in extracellular inulase activity (144 hours) was attained. Extracellular inulase activities attained at this time were 0.38 and 0.39 units/mg for 0.5% and 1.5% Tween 80, respectively. Towards the latter stages of the fermentation, the activity once again decreased perhaps as a result of the near exhaustion of inulin and/or the effect of stationary growth. Reese and Maguire (1969) reported that the role of the surfactant appeared to increase cell permeability and/or stimulate enzyme production. In Figures 11 and 13, it was observed that Tween 80 altered the disposition of the inulase enzyme when compared to the control fermentation. It appears that fermentations containing the surfactant generally showed a higher ratio of extracellular inulase activity in the medium to the amount of intracellular inulase activity associated with the cells. The presence of high levels of extracellular activity in the early stages of the fermentation would thereby enhance inulin hydrolysis in the medium. As a result (Figures 11 and 13) repressive effects on both intracellular and extracellular inulase synthesis became evident by the downward trend of inulase activity between 24 to 96 hours. As the fermentation progressed the demand for easily assimilable carbohydrates should be increased, diminishing the repressive effects on inulase synthesis such that the remaining inulin can be hydrolyzed into a simple carbohydrate source, presumably mainly consisting of fructose. This would appear to be true since both intracellular and extracellular inulase production rapidly increased when the culture fermentation was in the early stages of stationary growth

as an attempt to procure additional sources of energy for sustaining growth. The pattern of extracellular inulase shown in Figure 12 could not be explained despite similar duplicate results.

It would appear from the data that Tween 80 is not capable of stimulating inulase production but rather alters the localization of the enzyme in the fermentation. The data obtained from this study is considered to be highly inferring due to underlying factors of an uncontrollable nature which have altered the uniformity of inulase activity patterns, particularly so with extracellular inulase. Panchal and Stewart (1980) stated that although Tween 80 modifies the permeability of yeast cell membranes, there was both a rapid efflux and influx of organic molecules reported. In conjunction, the effects of low temperatures regarding increased cell membrane rigidity have been well documented. Therefore, alterations of normal inulase patterns could be the result of a combination of (i) effects of Tween 80 on the permeability, (ii) the exit and re-entry ratio of inulase and (iii) the effect of low temperature on permeability.

Effect of Growth Temperature on Inulase Production

The effects of temperature on the growth and production of inulase at 10, 20 and 30°C by C. kefyr are illustrated in Figures 14, 15 and 16. During the first 24 hour period the incubation temperature markedly affected the rate of biomass accumulation — 3.6 (10°C), 5.3 (20°C) and 5.8 mg/ml (30°C). Biomass accumulation was shown to increase steadily at 10°C, entering into an apparent stationary growth phase at 120 - 144 hours. When the temperature was increased to 20°C, the maximum biomass accumulation was noted at 24 hours, thereafter maintaining a steady-state growth pattern. A further increase in growth temperature to 30°C saw an initial peak in biomass at 24 hours (5.8 mg/ml) followed by a secondary rise (7.4 mg/ml) at 168 hours. The growth pattern exhibited at 30°C appeared to be diauxic. That is, the availability of certain substrates may lead to repression of the enzymes for metabolism of the other substrates. It is possible in this instance, that only when the concentration of the repressing substrate has been reduced through microbial assimilation can the enzyme for metabolism of other substrates be elaborated. Such regulation of microbial physiology leads to an aberrant growth cycle which shows one or more intermediate but transient stationary phases. Beluche et al. (1980) also reported that a D. cantarellii culture exhibited a diauxic effect when grown at 28°C, the fermentation was shown to completely utilize the simple monosaccharides before metabolizing inulin, at which time inulase activity became apparent. In this study during the

FIGURE 14. Effect of Growth Temperature on Inulase Production at 10°C.  , dry cell weight (mg/ml); , intracellular inulase (units/ mg protein); , extracellular inulase (units/ mg protein); , pH of culture medium.

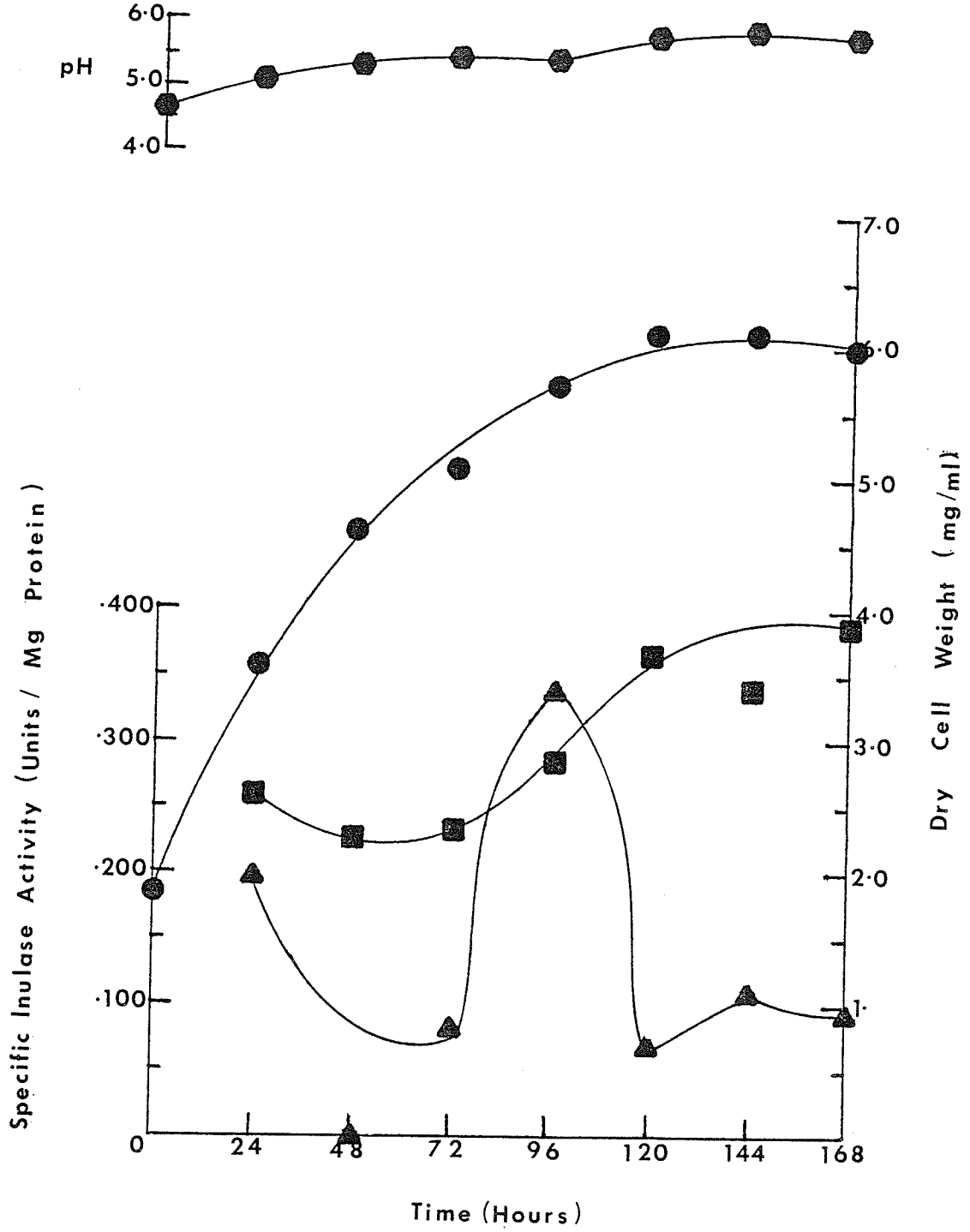
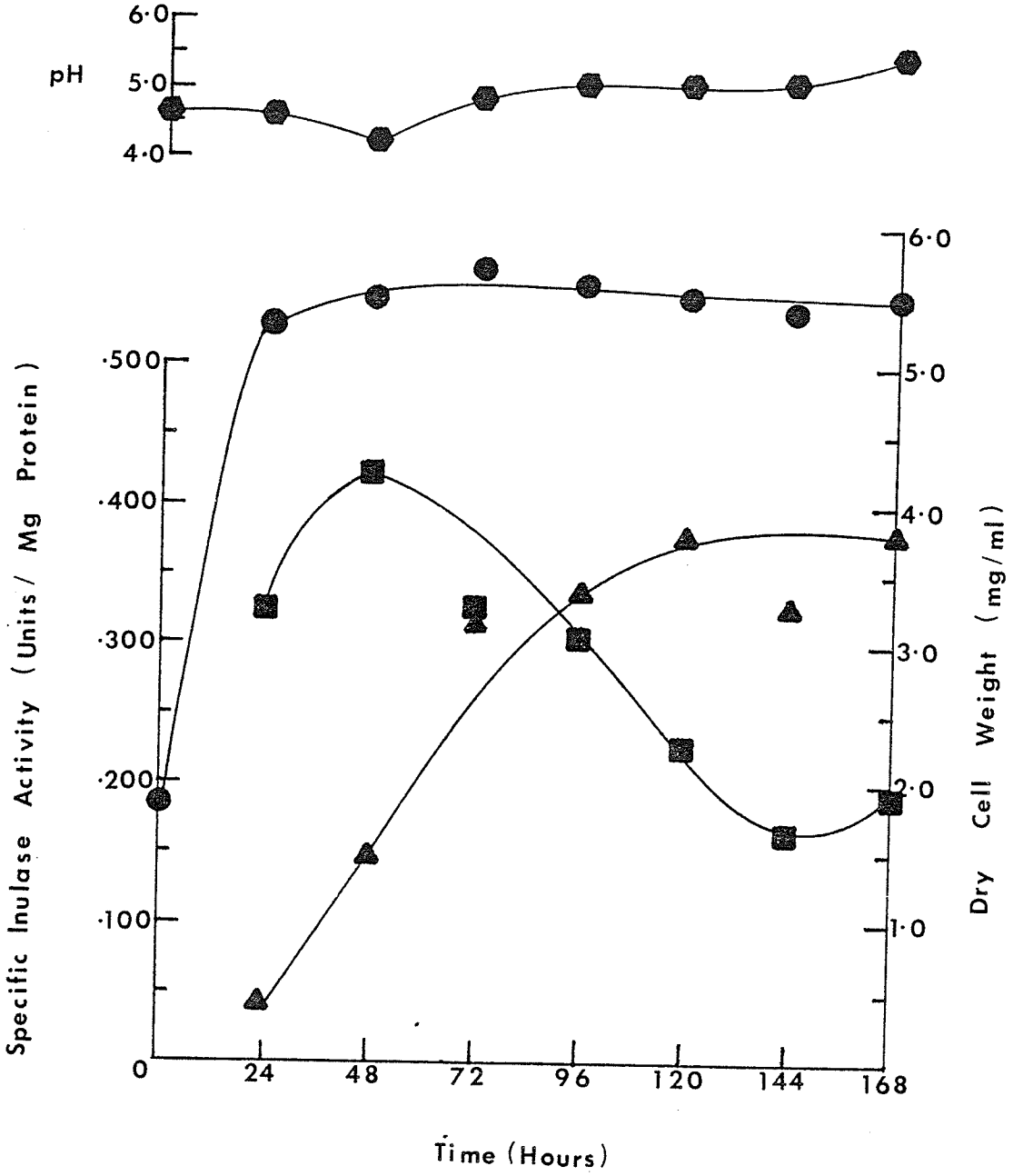

pH
6·0
5·0
4·0
Specific Inulase Activity (Units / Mg Protein)
·400
·300
·200
·100
0
Dry Cell Weight (mg/ml)
7·0
6·0
5·0
4·0
3·0
2·0
1·
24
48
72
96
120
144
168
Time (Hours)

FIGURE 15. Effect of Growth Temperature on Inulase Production at 20°C. ●—● , dry cell weight (mg/ml); ■—■ , intracellular inulase (units/ mg protein); ▲—▲ , extracellular inulase (units/ mg protein); ⬢—⬢ , pH of culture medium.


pH
6·0
5·0
4·0
Specific Inulase Activity (Units / Mg Protein)
·500
·400
·300
·200
·100
0
Dry Cell Weight (mg/ml)
6·0
5·0
4·0
3·0
2·0
1·0
24
48
72
96
120
144
168
Time (Hours)

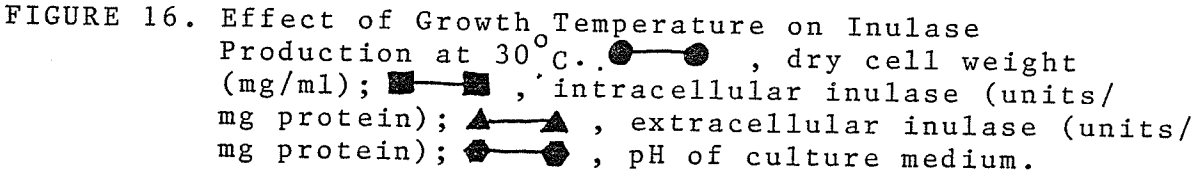
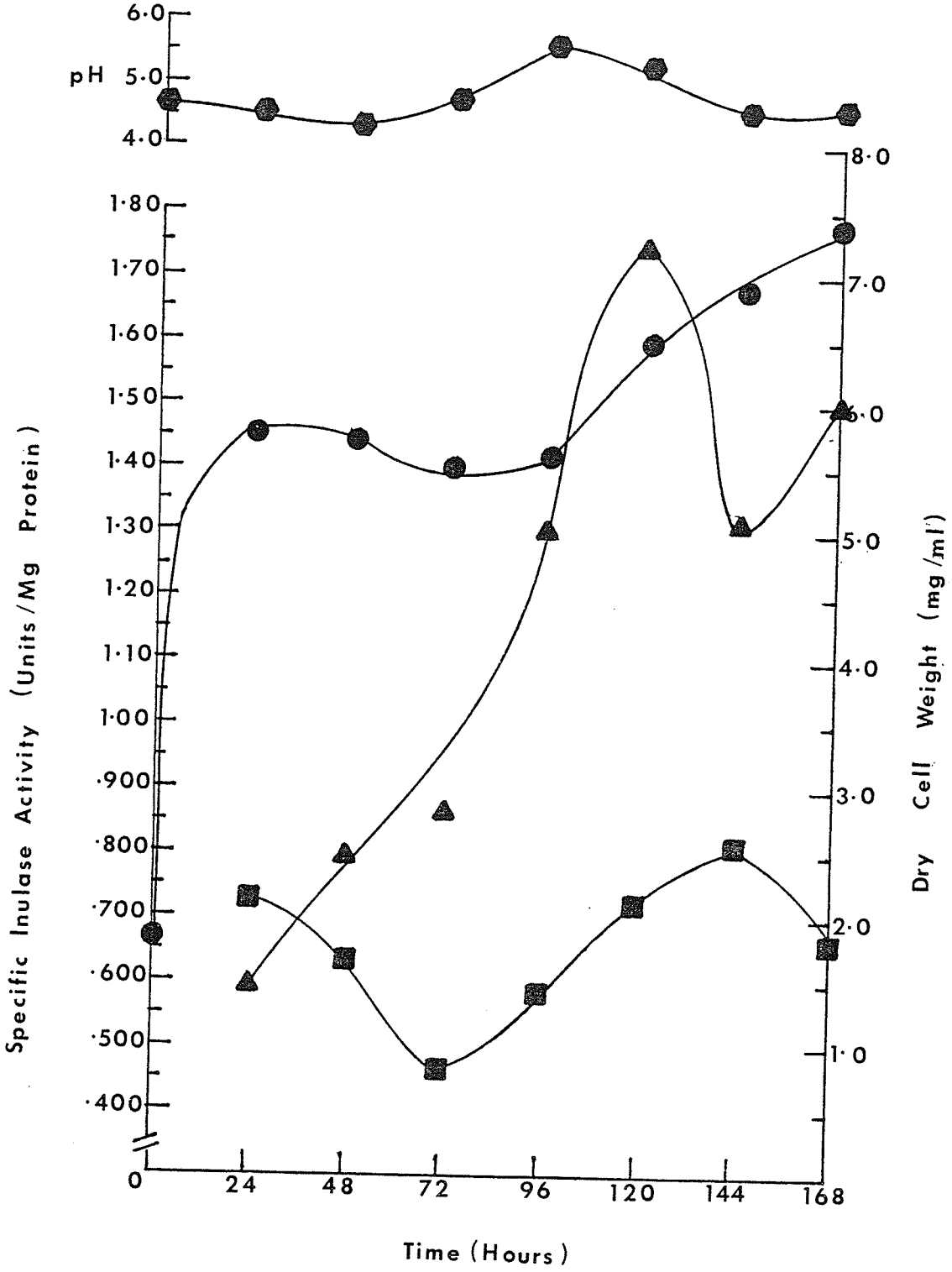


FIGURE 16. Effect of Growth Temperature on Inulase Production at 30°C. ●—● , dry cell weight (mg/ml); ■—■ , intracellular inulase (units/ mg protein); ▲—▲ , extracellular inulase (units/ mg protein); ⬢—⬢ , pH of culture medium.


6·0
pH 5·0
4·0
8·0
1·80
1·70
7·0
1·60
1·50
6·0
1·40
1·30
5·0
1·20
1·10
4·0
1·00
·900
3·0
·800
·700
2·0
·600
·500
1·0
·400
0
24
48
72
96
120
144
168
Specific Inulase Activity (Units/Mg Protein)
Dry Cell Weight (mg/ml)
Time (Hours)

first 24 hour growth period at 30°C, it can be assumed that the rapid growth progressed concomitant with a rapid utilization of simpler available carbohydrates (Figure 16). Upon the depletion or near depletion of these sugars, growth decreased, and an apparent stationary phase was reached. It is assumed that this growth phase was limited to the available carbon source. During this period, extracellular inulase was shown to increase in activity up until 96 hours of growth. This increase in extracellular inulase activity was followed by renewed growth, presumably due to an increase in simpler carbohydrates. It would appear that the stationary phase exhibited in this fermentation was partially a period of inulase induction and/or a relaxation of catabolite repression. The decrease in the intracellular level of inulase noted during the 24 - 72 hour period was shown to generally coincide with the apparent lag (stationary) period of growth (Figure 16). It is quite possible that part of the extracellular inulase activity increase observed during the same time period may in fact be intracellular inulase liberated during cellular lysis and/or protoplast formation. The increase in intracellular inulase (72 - 144 hours) would be expected if one assumed that the more easily available carbohydrates were near depletion, and that inulin was now the main carbon source. The length of the lag phase exhibited by this fermentation (24 - 96 hours) was shown to be quite long. It is conceivable that although inulase was being synthesized, the levels of the enzyme were still insufficient to cause rapid hydrolysis of inulin in order to support an exponential growth. Up until 96 hours,

it is possible that the inulase synthesized by the organism was providing the fermentation with only enough fructose to maintain steady-state respiration (rate limiting) and that only after 96 hours did the extracellular inulase activity reach high enough levels in order to provide an adequate level of available carbohydrate (fructose). Further evidence for this possibility is the concomitant increase in intracellular inulase activity.

The diauxic effect noted in the 30°C fermentation was absent when carried out at 20°C. It was thought that the lack of this diauxic effect was indirectly attributed to the temperature used. Since the biomass accumulation was lower than that observed at 30°C, it is possible that the population pressure placed upon the substrate was not as great as at 30°C. That is, although the extracellular inulase activity was shown to increase in the early stages of the fermentation, the activity was not sufficient to provide adequate levels of readily assimilable carbohydrates for active growth. It is thought, rather, that the extracellular inulase activity produced in this fermentation was sufficient only to provide enough simple carbohydrates to sustain respiration and/or steady-state conditions. It is also believed that the extracellular inulase activity may be partially or totally intracellular inulase activity which was liberated by cellular lysis and/or protoplast formation. The intracellular inulase increase in production from 24 to 48 hours (Figure 15) was most likely due to the rapid growth rate causing an accelerated

depletion of carbohydrates in the medium. This would also seem plausible if one takes into account the repressive effect of easily assimilable carbohydrate sources i.e. as simple carbohydrate sources are utilized, the repressive effects will become diminished. The corresponding decreases in intracellular inulase can be explained by steady-state conditions and by renewed availability of easily assimilable carbohydrates, possibly fructose.

The effect of temperature on growth appeared to influence rate of inulase production. Since growth at 10°C is sub-optimal, the rapid utilization of easily metabolizable sugars does not occur and as such it is only in the latter periods of growth that a stationary phase is observed. As the temperature is increased, it is presumed that the growth rate is increased, thereby a stationary phase of growth is encountered much earlier in the fermentations at higher temperatures. It has already been noted that lowering the temperature below the optimum decreases the rate of growth of a batch culture of a microorganism. The effect of the growth temperature on the site and/or location of inulase synthesis cannot be demonstrated since protoplast and/or cellular lysis would alter the disposition of inulase (Table 3). That is to say, the intracellular inulase that is being released into the medium would thereby give a false impression of the true level of extracellular inulase activity which is actually present within the medium.

The implications of this study reveal that additional studies are necessary to characterize the true extracellular

TABLE 3. Disposition of Intracellular and Extracellular Inulase Activity Under Varying Growth Temperatures.

	10°C			20°C			30°C		
Time (hrs)	Total[a] Inulase	% Extra	% Intra	Total[a] Inulase	% Extra	% Intra	Total[a] Inulase	% Extra	% Intra
24	0.456	42.8	57.2	0.358	9.8	90.2	1.324	45.1	54.9
48	—[b]			0.568	25.5	74.5	1.432	55.6	44.4
72	0.319	26.6	73.4	0.651	49.9	50.1	1.332	64.9	35.1
96	0.618	53.9	46.1	0.642	52.2	47.8	1.883	69.0	31.0
120	0.432	15.5	84.5	0.608	62.3	37.7	2.469	70.5	29.5
144	0.448	24.1	75.9	0.496	65.9	34.1	2.128	61.6	38.4
168	0.477	19.5	80.5	0.576	66.1	33.9	2.179	69.3	30.7

a Total inulase is expressed as specific inulase activity (units/mg protein). The total inulase is the combined intracellular and extracellular inulase activity.

b The data obtained for this time period was considered to be inaccurate due to faulty analysis.

to intracellular inulase ratio synthesized by the yeast cell. A method used by Arnold (1980) was capable of distinguishing between enzymes that are associated with the cell wall and those located within the periplasmic space. The periplasmic space is identified as the shell which is bound by both the cytoplasmic membrane and the outer rigid cell wall. The technique used involves the use of a French pressure device which ruptures the cell wall thereby allowing the periplasmic enzyme to be released while homogenization is required to release cell wall associated enzymes. If the enzyme in question is indeed located within the periplasmic space, it should be co-released with an already characterized periplasmic enzyme. Secreted enzymes (extracellular) were identified from cellular enzymes by their resistance to centrifugation and filtration.

SUMMARY AND CONCLUSIONS

Inulase (β-fructofuranosidase) was synthesized by the mesophilic yeast, Candida kefyr at sub-optimal temperatures. The pattern and localization of intracellular and extracellular inulase was investigated. The findings from this investigation are as follows:

1. Inulase production by C. kefyr at 10°C in a starch lactose inulin based medium showed maximum production at 144 hours which coincided with stationary growth of the organism. Both the intracellular and extracellular inulase were repressed in the early stages of the fermentation likely due to catabolite repression.
2. Increasing the initial inoculum of the fermentation did not significantly decrease the repression of inulase. Higher levels of inulase, however, were achieved most probably due to the higher biomass accumulation.
3. The addition of yeast extract does affect inulase production and optimally promoted intracellular inulase activity using .35% w/v. Yeast extract concentrations of 0.17% and 0.53% did not increase inulase production. Yeast extract at the higher concentration was shown to be inhibitory towards inulase production or alternately interfered with enzyme expression.
4. Increasing the rate of agitation from 150 to 300 rev min^{-1} increased total inulase production from 0.94 to 1.28 units/mg, respectively. This increase in inulase production was accompanied with a slight increase in biomass.

Agitation at 500 rev min^{-1} accompanied by air sparging (1.5 ml/min/ml fermentation medium) was shown to be deliterious towards extracellular inulase activity. Susceptibility of the enzyme to surface denaturation caused by the gas - water interface is indicated.

5. The addition of a surfactant (Tween 80) to the fermentation did not show any stimulatory effects on inulase production. Levels of extracellular inulase were generally increased as surfactant concentrations were increased.

6. Inulase production at 30°C showed highest levels of total inulase, in addition the effect of catabolite repression was not predominant at this temperature as compared to 20 and 10°C. The reduction in catabolite repression at 30°C was presumably due to the high population pressure placed on the substrate. A diauxic growth effect at 30°C was evident.

Cultivation of C. kefyr at sub-optimal temperatures appeared to increase the ratio of intra to extracellular inulase perhaps due to restricted cellular lysis and/or altered membrane - cell wall flux. However, sub-optimal temperatures did not increase inulase yields. This is contrary to the findings by several researchers where greater extracellular enzyme yields were obtained at temperatures lower than the optimum growth temperature (Buehler et al., 1951; Nashif and Nelson, 1953; Pan et al., 1950). The microorganisms investigated in these studies were not performed with inulase.

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APPENDIX

Unit Abbreviation	
Å	Angstrom
g	Gram
mg	Milligram
min	Minute
ml	Millilitre
M	Molar concentration
N	Normal concentration
rev min^{-1}	RPM
um	Micrometer
µ mole	Micromole
µg	Microgram
x g	Gravitational acceleration