

Xylose and Xylose/Glucose Fermentation by Yeast: A Strain Comparison

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Abstract

The ability of 5 strains of xylose fermenting yeast to convert xylose and xylose/glucose mixtures to ethanol were compared. *Pichia stipitis* NRRL 7124, *Pichia stipitis* NRRL 11545, *Pachysolan tannophilus* NRRL 2460, and *Pichia segobiensis* NRRL 11571 and Tygel 12 from Xylan, Inc. It was found that *Pichia stipitis* 11545 was the best performing xylose/glucose to ethanol strain in these experiments. *Pichia stipitis* 7124 and 11545 performed similarly on 4 and 6% xylose fermentations with both strains able to produce about 20 g/l ethanol on a pure xylose feed, but strain 11545 showed substantially better performance on mixtures of glucose and xylose. Strain 11545 was able to produce up to 27 g/l ethanol on mixtures of glucose and ethanol. Xylose was not utilized in glucose/xylose mixtures until the glucose level was less than 15 g/l. Yeast extract was shown to speed the fermentation as compared to corn steep. *Pichia segobiensis* 11571 showed stronger ethanol inhibition than the two *Pichia stipitis* strains, with fermentation ceasing at 10 to 11 g/l., while Tygel 12 showed good growth on xylose, but no measurable ethanol production in semi-anaerobic fermentation mode was noted.

Introduction

There is a great deal of interest in converting wood and biomass streams to renewable fuels. The basic composition of these ligno-cellulosic materials is a mix of three major components, cellulose which breaks down to glucose, hemicellulose which breaks down primarily to xylose, and lignin. The economics of biomass conversion to ethanol are improved if both the hemi-cellulose and the cellulose are converted to ethanol (Ladisich and Swartzkopf, 1993). The conversion of xylose to ethanol is being intensively studied with a variety of strains found to be able to efficiently convert xylose to ethanol. These strains include *Pichia stipitis*, *Pachysolan tannophilus*, *Candida shehatae*, and genetically modified yeast and bacteria. Thermophilic anaerobic conversion of

cellulose to ethanol by *T cellulum* has been demonstrated by several researchers (Zertuche and Zall, 1982; Shahbazi et al, 1991) but this organism is only able to produce 5 to 7 g/l or less of ethanol, and generally produces a number of side products (organic acids). The conversion of xylose to ethanol by *Pichia stipitis* has been recently discussed by Laplace et al (1991) who compared *Pichia stipitis* 7124 and a strain of *Candida shehatae*. They found 7124 gave a better conversion efficiency ($Y_{p/s}$ of 0.44) as compared to the *Candida shehatae* (0.40) with both strains giving similar maximal ethanol concentration of about 30 g/l. Grootjen et al (1990) determined a maximal ethanol productivity of 0.13 g eth/g cell hr for *Candida shehatae* CBS 5773. They determined that there was a maximal yeild ($Y_{p/s}$) of 0.31 g eth/g xylose when no oxygen was added to the fermenter, with yeild then falling to zero ethanol if oxygen at 0.03 moles/l*hr was added to the fermenter (the cells going to an aerobic biomass production mode). Dominguez et al (1993) determined maximal growth rates (μ_{max}) and substrate affinity constants (K_s) from the Monod model for *Pichia stipitis* 7124 under aerobic and anaerobic environments. Values of 0.19 g/g*hr were determined for specific growth rates under aerobic conditions as compared to 0.066 g/g*hr under anaerobic conditions. Guebel et al (1991) also determined that growth was very slow under anaerobic conditions for *Pichia stipitis* 7124, while at 5 mmol O₂/l*hr, a maximal growth rate of 0.11 g/g*hr was noted. An ethanol yeild of .34 g ethanol/g xylose was measured under anaerobic conditions, while at 5 mmol O₂/l*hr, yield fell to 0.28 g/g. The effects of combined glucose and xylose mixtures on *Pichia stipitis* behavior has been studied by Grootjen et al (1991). It was determined that with a 40 g/l glucose and 10 g/l xylose, the *Pichia stipitis* would preferentially metabolize glucose with only 20% of the xylose utilized (either for cell growth or ethanol production). A steady state ethanol concentration of 14 g/l was attained in a CSTR. It was determined from this experiment that simple utilization of the glucose by a *Saccharomyces cerevisiae* yeast would give comparable or better results.

The effects of pH and temperature on *P stipitus* 7124 growth and productivity was evaluated by Slininger et al(1990). A wide optimum for pH between 4 and 7 and for temperature of 25 to 33 for cell growth was noted, with a μ_{max} of 0.55 g/g h. Similarly, for productivity, a v_{max} of 0.16 and P_m of 45-50 (30°C) to 55 (25°C) was noted between pH 4 and 6. Maximum yields ($Y_{p/s}$) of about 0.37 at 25°C can be determined from data presented. Growth rates and ethanol tolerances were higher in this study than in other reports, perhaps due to improved oxygen transfer rates.

Genetically engineered *Saccharomyces cerevisiae* able to ferment xylose have been reported by Tantirungkij et al (1993) who were not able to actually generate much ethanol, and Ho et al (1994) who seems to have successfully completed a fermentation of both glucose and xylose with the transformed yeast. A recombinant *E. coli* has been shown to be able to ferment xylose well, with optimal pH of 6 to 7 (Bealle et al, 1991). Actual fermentation of biomass hydro-

lysate has not been able to duplicate these results, with ongoing experiments trying to determine the components poisoning the fermentation (Bothast, 1994).

The basic purpose of this study was to compare some of the better strains of xylose to ethanol yeast.. We are investigating a process in which hemicellulose/lignin are first separated and hydrolysed based on the Xylan process (Tyson et al, 1993). In this process biomass is mechanically extruded at high temperatures and pressures in the presence of some chemicals. Carr and Doane (1984) reported on the use of a similar process for straw pretreatment. This Xylan Delignification Process (XDP) causes a breakdown of the lignin and hemicellulose which can then be separated from the cellulose by rinsing. Fermentation of the xylose can then occur prior to cellulose fermentation. This pre-saccharification of xylose eliminates the problem of trying to ferment xylose in the presence of glucose. Gorton et al (1991) suggests a similar strategy following the poor results noted in his co-fermentation experiments.

Methods

Four strains of xylose fermenting yeasts were obtained from C. Kurtzman of the NCAUR (*Pichia stipitis* NRRL 7124, *Pichia stipitis* NRRL 11545, *Pachysolen tannophilus* NRRL 2460 and *Pichia segobiensis* NRRL 11571), and one strain from Xylan Inc. (Tygel 12). Each of these strain was grown up aerobically (1 vvm air through an airstone sparger, 300 ml broth in a 500 ml erlemyer flask) at 25 C. in a media consisting of 20 g/l xylose and 20 g/l corn steep liquor. The final cell densities ranged from 9.5 to 11 g/l dry basis. These cells were separated from the cell broth and added to the test fermentation media at a cell concentration of 5 to 7 g/l cells. Test fermentations of 60 ml were performed in 150 ml flasks held in an incubator at 30 C. Each flask was stirred at 180 RPM using a magnetic stirrer/stir bar. The flasks were capped with a foam plug covered with foil. A sulfite oxidation test (ref) indicated an oxygen tranfer rate of 8.75 mmol/l h under these conditions.

The standard test medium consisted of the test sugar(s) and a nutritional supplement of 8 g/l yeast extract. Some of the latter experiments tested the effects of different nutritional supplements with the effects of yeast extract compared to corn steep liquor. Ethanol, xylose, and co-product changes in concentration were monitored by analysing samples from the batch reactions in a HPLC fitting with an HPX87H aminex column (Biorad) and a refractive index detector.

Results

The basic fermentation performance of *Pichia stipitis* 11545, *Pichia stipitis* 7124, *Pichia segobiensis* 11571 and *Pachysolen tannophilus* 2460 on the fermentation of a 35 to 40 g/l xylose medium are shown in Figures 1 through 4. It can be seen that *Pichia stipitis* 11545 and 7124 fermented the xylose quite

effectively. An ethanol yield ($Y_{p/s}$, gm ethanol per gm xylose) of 0.46 and 0.36 were determined respectively. *Pachysolen tannophilus* 2460 (Figure 3), however, showed substantial quantities of xylulose co-product ($Y_{co-p/x}$ of 0.35) reducing the ethanol yield coefficient to 0.29. Fermentation of 60 g/l xylose was next examined as shown in Figures 5 through 8. *Pichia stipitis* 11545 slightly outperformed *Pichia stipitis* 7124 in this experiment, with strain 11545 dropping the sugar concentration to 21 g/l in 120 hours, while strain 7124 dropped the xylose to 25 g/l. Strain *Pichia stipitis* 11545 also gave a better yield than 7124 at this higher sugar concentration, (0.46 versus 0.43 g/g). *Pachysolen tannophilus* 2460 showed a considerably poorer performance with only 14 g/l ethanol being produced in 120 hours, while *Pichia segobiensis* 11571 showed only 10 g/l in ethanol 120 h (Figure 7 and 8). In repeated trials, the yeast Tygel 12 showed good aerobic and anaerobic growth on xylose, but no measurable ethanol was produced in any trials.

The effects of glucose and xylose in the same fermentation mixture was studied. The four strains were fermented in a fermentation broth containing 50 g/l xylose and 40 g/l glucose. Strain *Pichia stipitis* 11545 showed preferential use of the glucose followed by a slow fermentation of the xylose as shown in Figure 9. Glucose was totally utilized within 50 hours. Xylose began to be fermented once glucose levels were under 15 g/l. A final ethanol concentration of 26 g/l was reached at 120 hours, with a yield, $Y_{p/s}$, of 0.35 g/g. Strain *Pichia stipitis* 7124, suggested by Laplace et al (1991) as a top performer, showed a poorer performance (Figure 10). Glucose and xylose seem to both be metabolized, but only very slowly. Glucose levels dropped from 43 to 14 g/l, xylose from 50 to 36 g/l, and ethanol increased from 0 to 12 g/l over 120 hours for a net yield ($Y_{p/s}$) of 0.28. *Pichia segobiensis* 11571 also first metabolized glucose, with all the glucose metabolized in 44 hours. At this point, some xylose began to be very slowly converted to ethanol. A higher ethanol level was developed in this mixed media (19 g/l) than was attained on xylose media (10-11 g/l). Strain *Pachysolen tannophilus* 2460 showed a strong preference for glucose as did *Pichia stipitis* 11545. As seen in Figure 12 glucose was utilized by 50 hours, and xylose dropped to 30 g/l by 70 hours, after which the fermentation ceased. A final ethanol concentration of 20 g/l was reached with a yield, $Y_{x/s}$ of .31 g/g. Interestingly, there was little production of xylulose during this co-sugar fermentation.

These experiments suggest that strain *Pichia stipitis* 11545 has definite performance advantages over the other 4 strains. In a pure xylose fermentation, *Pichia stipitis* 11545 and 7124 behave almost identically, while in a mixed sugar environment, *Pichia stipitis* 11545 definitely fermented more quickly than the 7124. Strain *Pachysolen tannophilus* 2460 showed a propensity towards xylulose production when fermented on pure xylose, but performed similarly to *Pichia stipitis* 11545 on mixed sugars, showing a better performance on glucose than on xylose. These trials suggest that strain 11545 is the best performing ethanol producer of the 5 strains of yeast examined for the

production of ethanol from xylose and xylose/glucose mixtures.. We therefore ran some further experiments on strain *Pichia stipitis* 11545.

The effects of various glucose/xylose levels on *Pichia stipitis* 11545 is shown in Figure 13. These experiments verified what was seen in our previous experiment (Figure 7), that xylose will be fermented when glucose levels are under 15 g/l. When glucose levels are over 15 g/l, very little xylose is fermented. High sugar levels are somewhat inhibitory, with the highest ethanol concentration (25 g/l) achieved from the fermentation of a mix of 60 g/l glucose, 30 g/l xylose (experiment 18). Xylose was only utilized in experiment 19 where initial glucose was 30 g/l, and experiment 16 with no glucose (95 g/l initial xylose). If initial fermentation rates are used to determine specific fermentation rate, n , g ethanol/ g cells per hour, a value of 0.068 can be determined from experiments at 4, 6, and 10% xylose. A similar rate was noted during the conversion of glucose in mixed fermentations (Figure 9). Ethanol inhibition seems complete at about 30 g/l, so a general model for the xylose fermentation with strain *Pichia stipitis* 11545 can be written as:

$$v = v_{\max} (1 - P/P_m)$$

where v_{\max} is 0.068, and P_m is 30 g/l. These values are considerably lower than values determined by Slininger et al (1990) of v_{\max} of 0.14, and P_m of 55 g/l for strain 7124. This is probably due to the addition of oxygen (K_{La} of 0.165/min) to the fermenter by this group, giving the higher rates but with a concomitant loss in yeild (0.37) due to cell growth during the fermentation.

Some nutritional experiments are shown in Figure 14, where various nutritional supplements were added to a 55 g/l xylose medium. Replacement of yeast extract with various levels of corn steep were not very successful. Very little difference in fermentation rates was noted at the three levels of corn steep added (0.33, 0.67, and 1% levels), with the lower level of corn steep actually fermenting a little bit faster than the higher levels. The addition of yeast extract (5 g/l) caused the fermentation to proceed more quickly (v of 0.045 g eth/g cell*h) than the fermentations supplemented with corn steep liquor (v of 0.023 g/g*h).

Conclusions

Strain *Pichia stipitis* 11545 was selected from among 5 strains of xylose metabolizing yeasts. This strain shows an ability to produce up to 3% ethanol from a 6% xylose or xylose/glucose mix. Specific productivity was determined to be about 0.042 g/g*h for this strain on xylose in a low oxygen transfer environment. A good conversion efficiency of 0.44 to 0.46 was shown on pure xylose substrates (85-90% of theoretical conversion). Strain *Pichia stipitis* 7124 performed similarly as strain 11545 on xylose, but showed considerable glucose inhibition in mixed sugar fermentations. Strain *Pichia segobiensis* 11571 showed

a good conversion efficiency but was more strongly ethanol inhibited with 10 to 11 g/l ethanol maximum ethanol on pure xylose. Strain *Pachysolen tannophilus* 2460 was a less effective xylose fermenter, producing approximated equal weights of xylulose and ethanol. Tygel 12 grew well on xylose, but produced no measurable amounts of ethanol.

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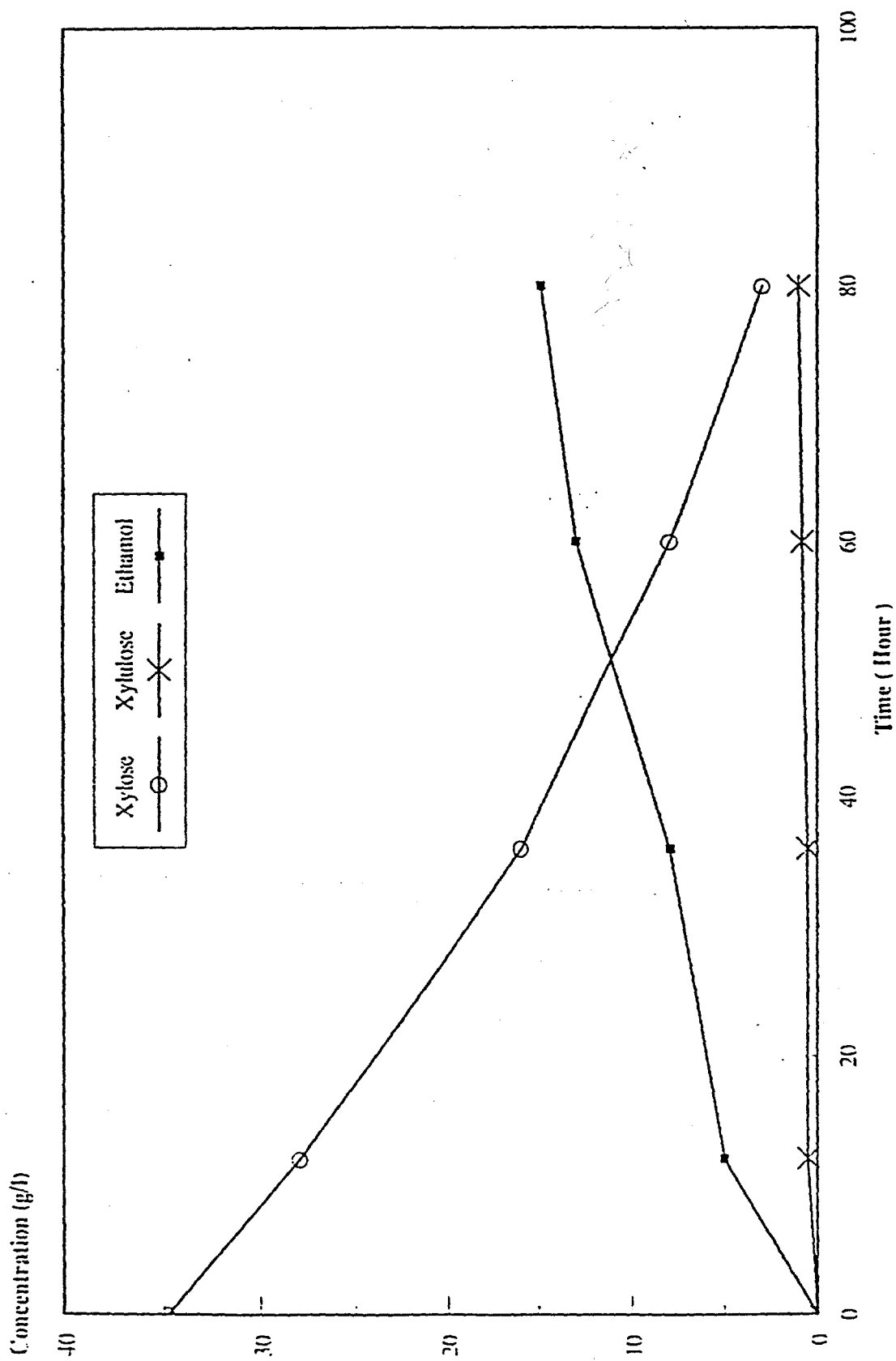


Figure 1. Fermentation of 4% D-Xylose with *Pichia stipitius* 11545

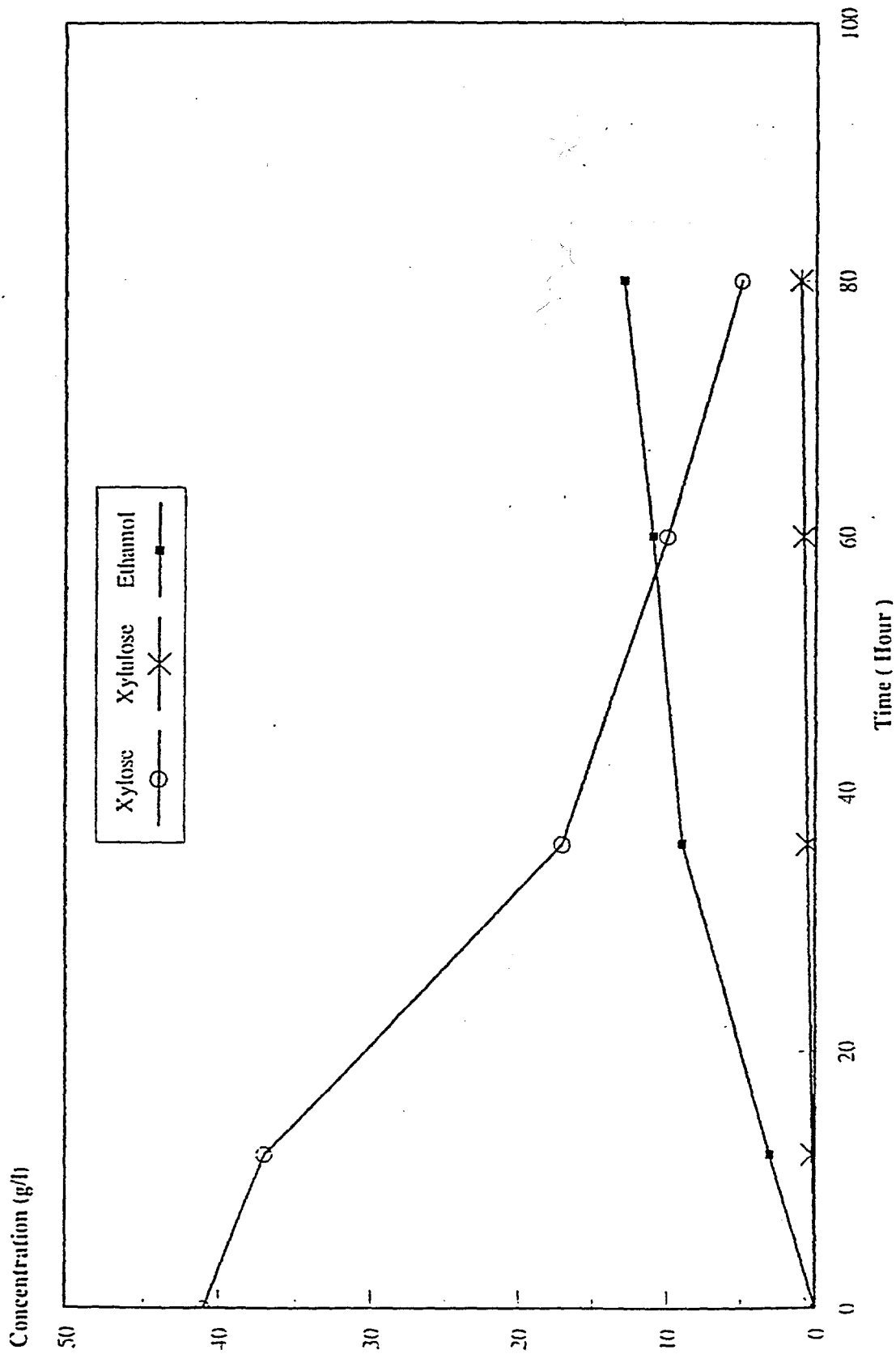


Figure 2. Fermentation of 4% D-Xylose with *Pichia stipitius* 7124

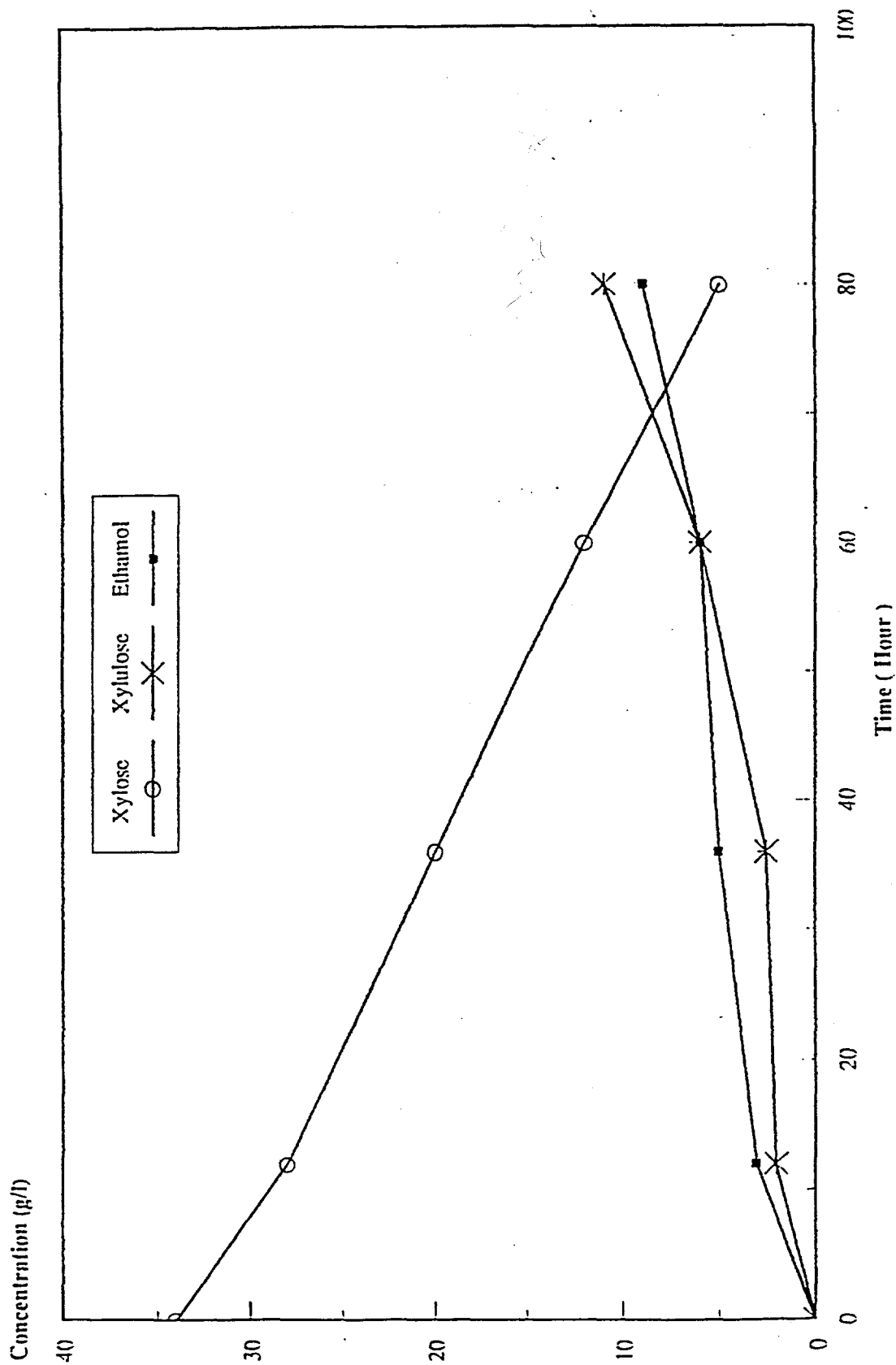
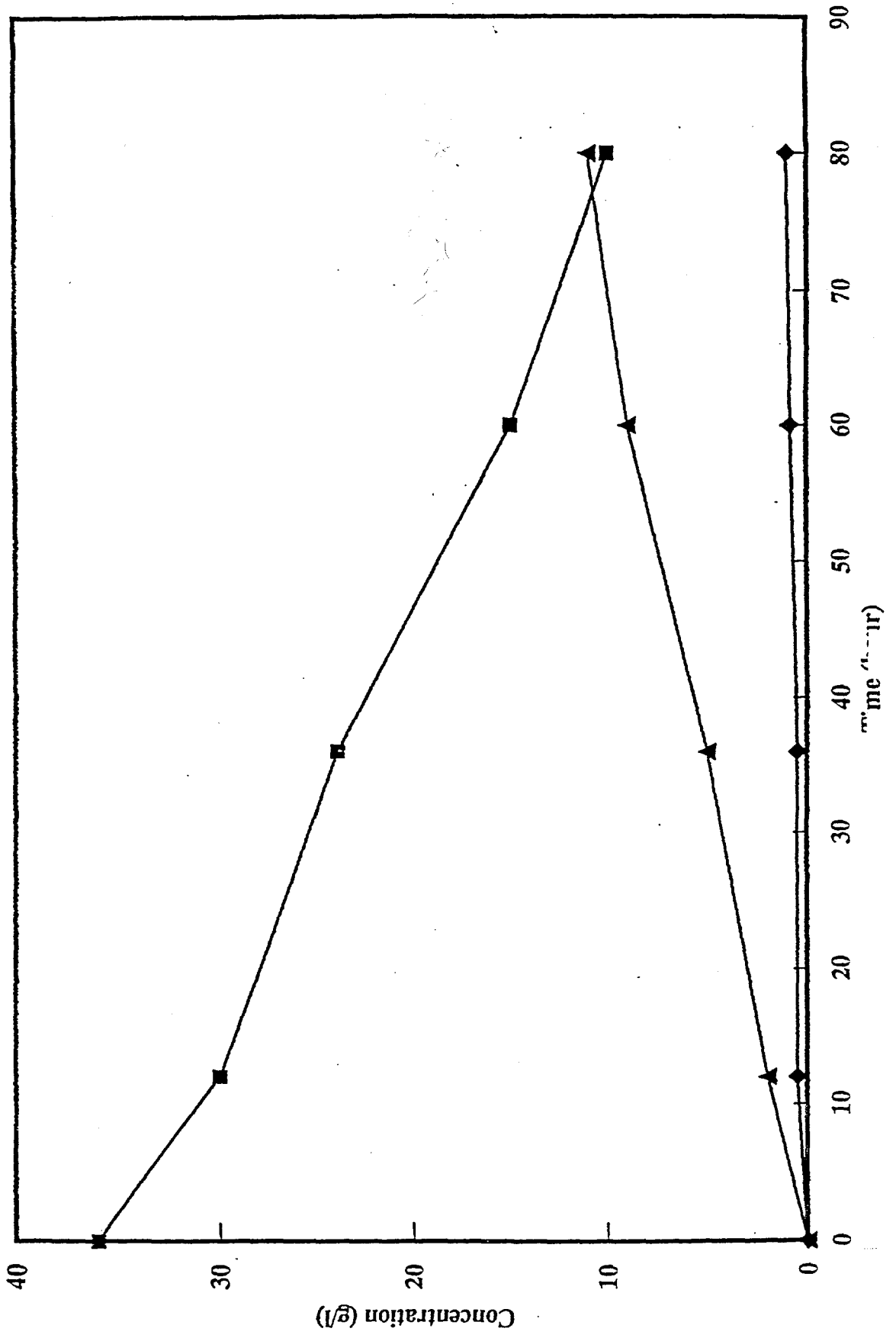


Figure 3. Fermentation of 4% D-Xylose with *Paclysolen tannophilus* 2460

Figure 4. Fermentation of 4% D-Xylose with *Pichia segobiensis* J1751



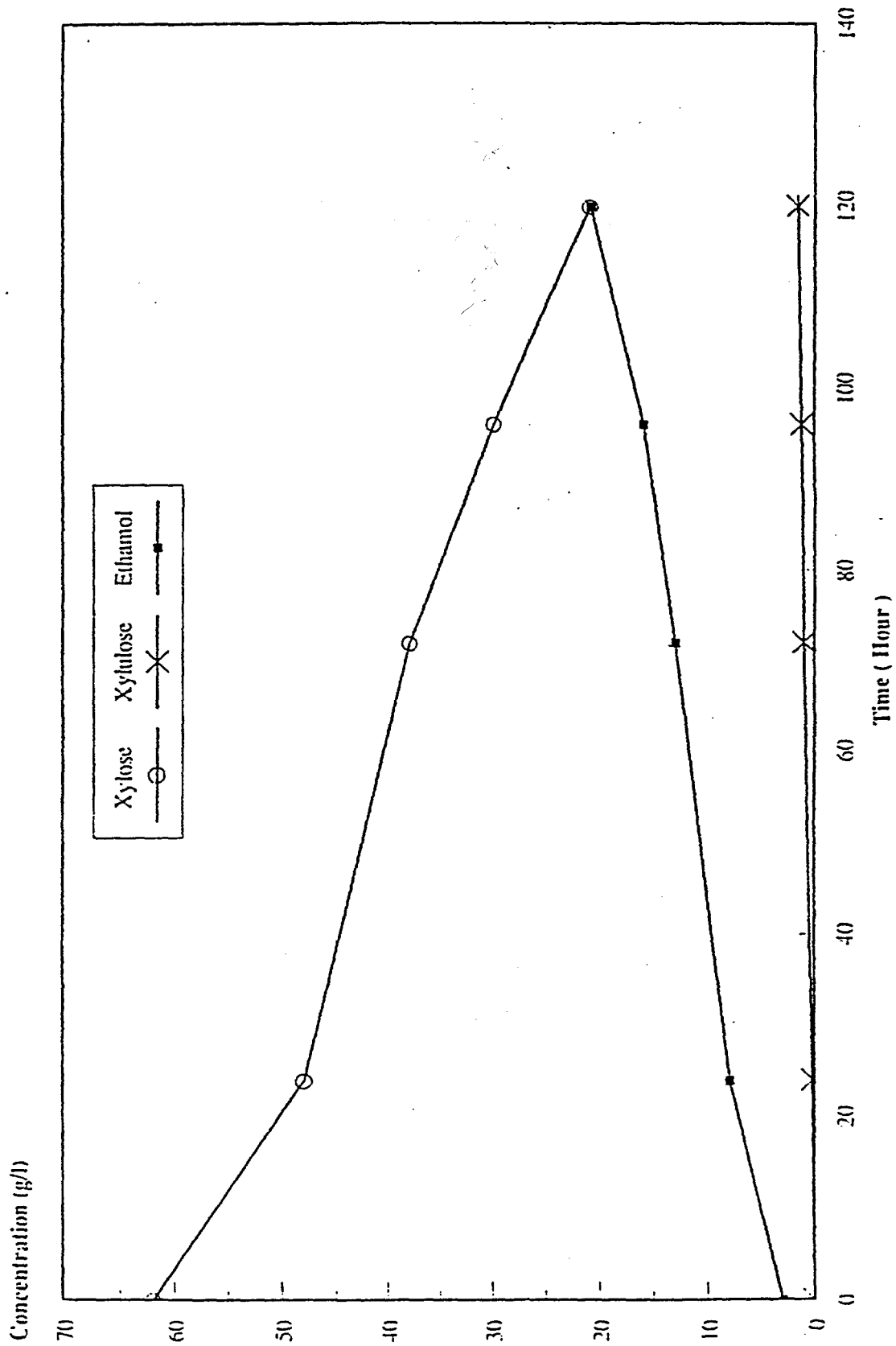


Figure 5. Fermentation of 6% D-Xylose with *Pichia stipitius* 11545

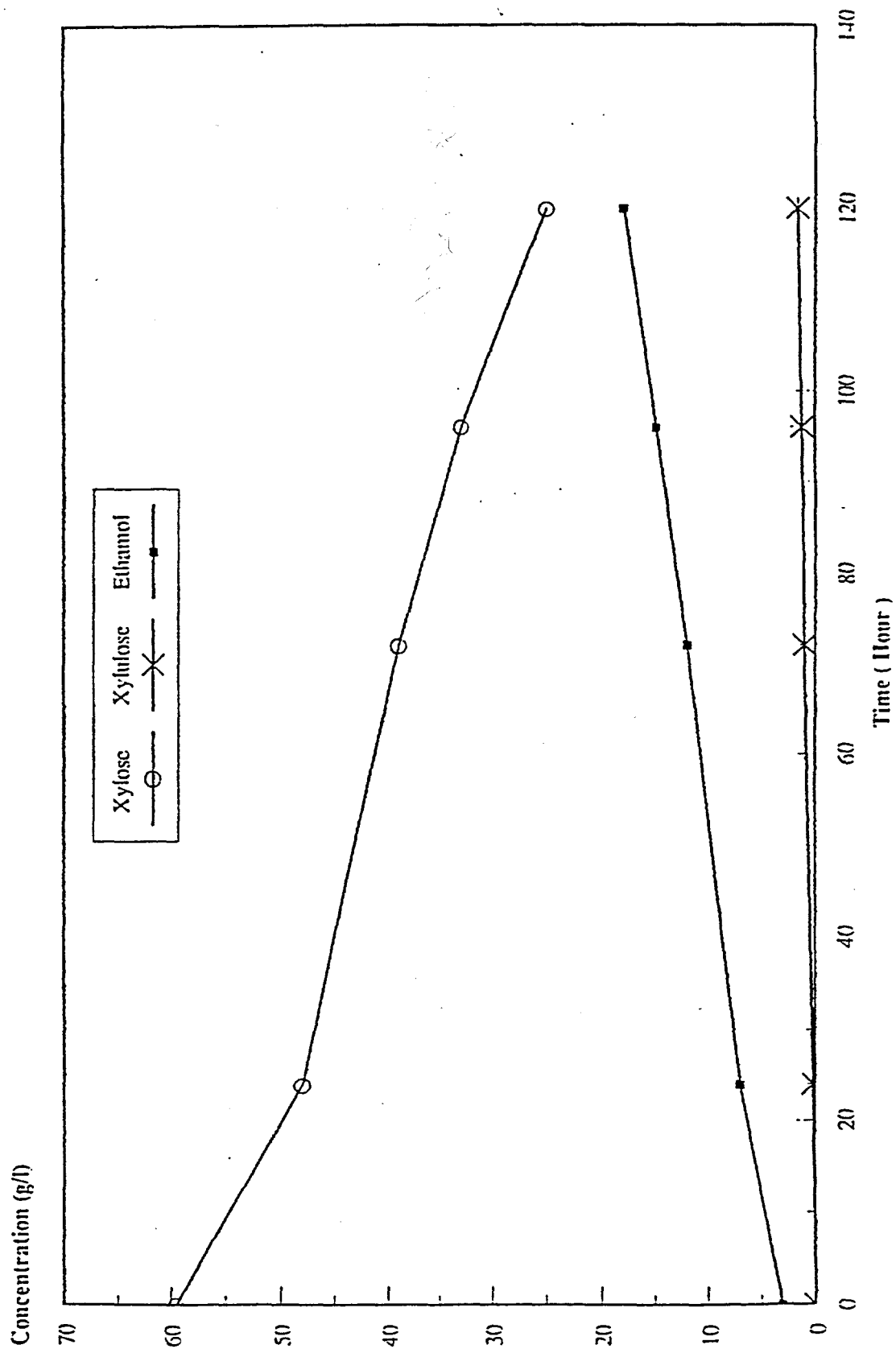


Figure 6. Fermentation of 6% D-Xylose with *Pichia stipitius* 7124

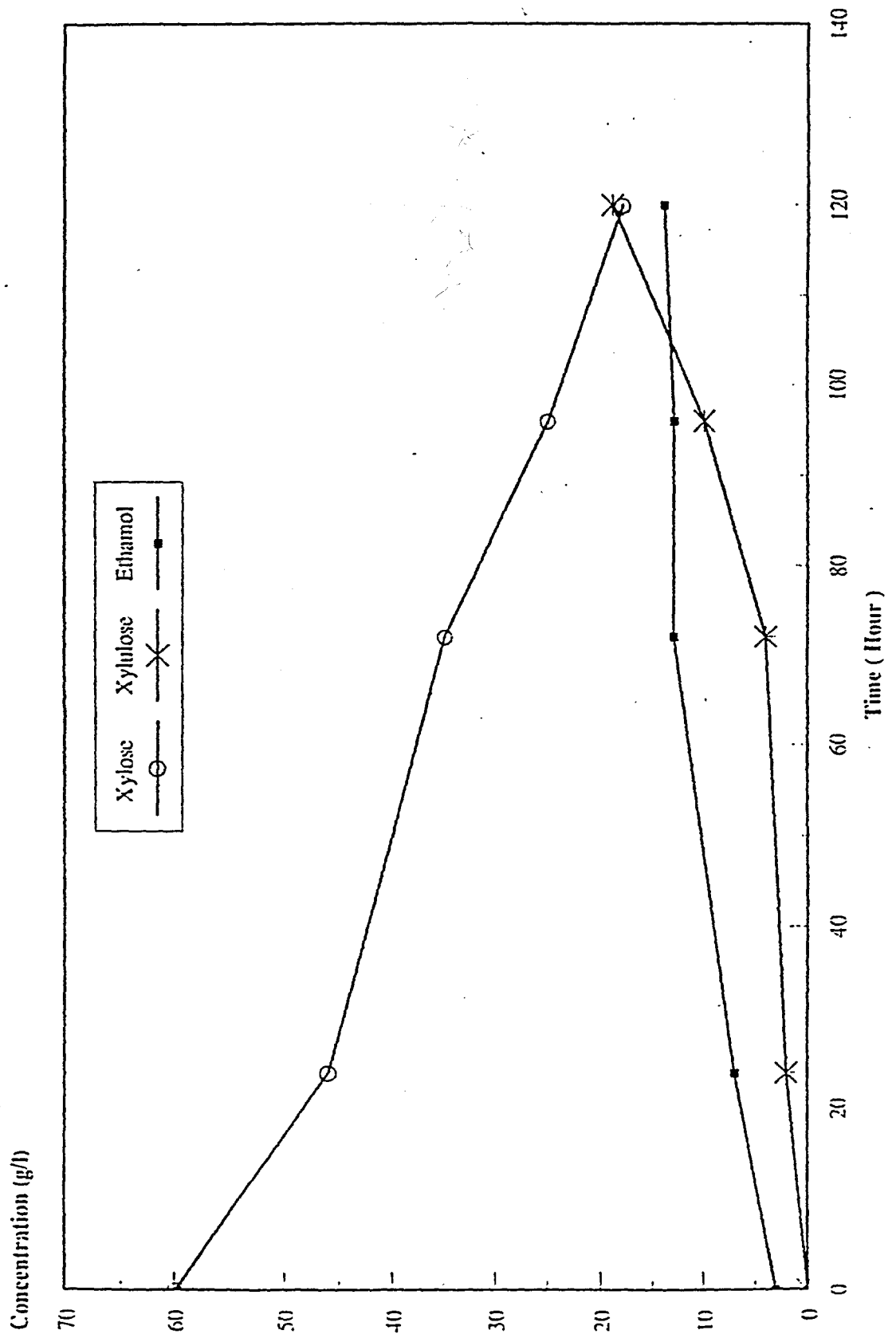
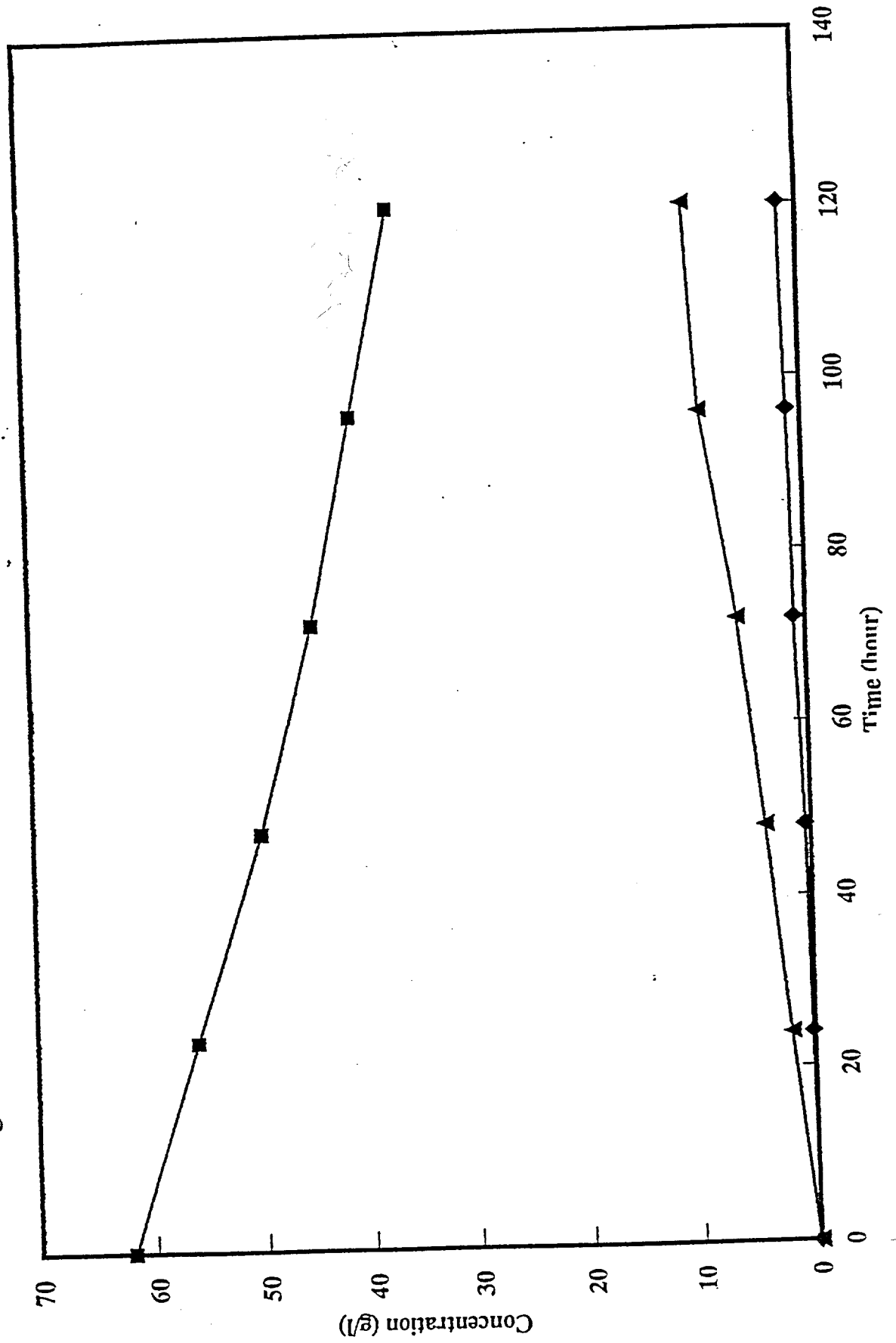


Figure 7. Fermentation of 6% D-Xylose with *Pachysolen tannophilus* 2460

Figure 8. Fermentation of 6% D-Xylose with *Pichia segobiensis* 11751



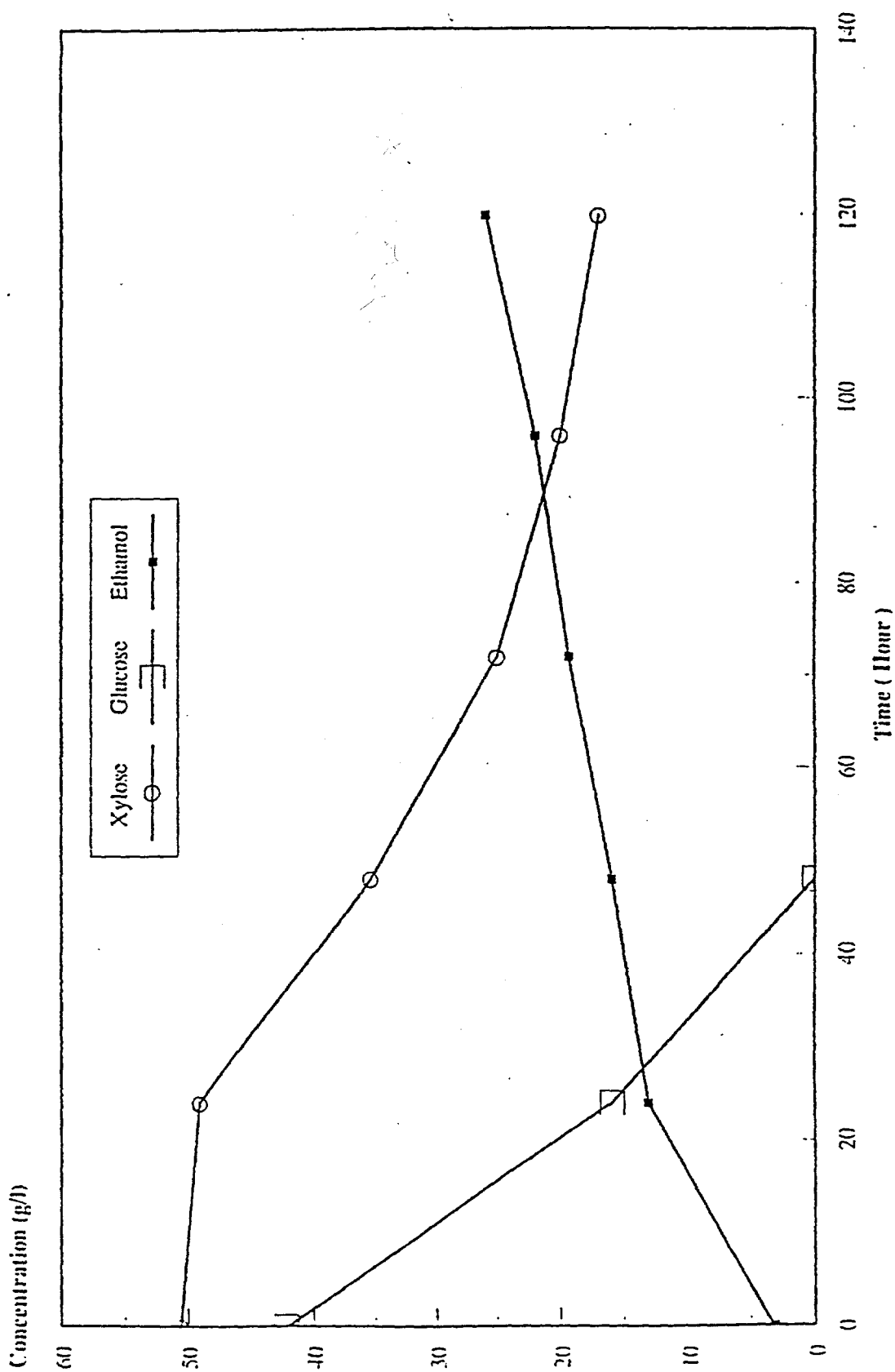


Figure 9. Fermentation of 4% Glucose/5% D-Xylose with *Pichia stipitius* 11545

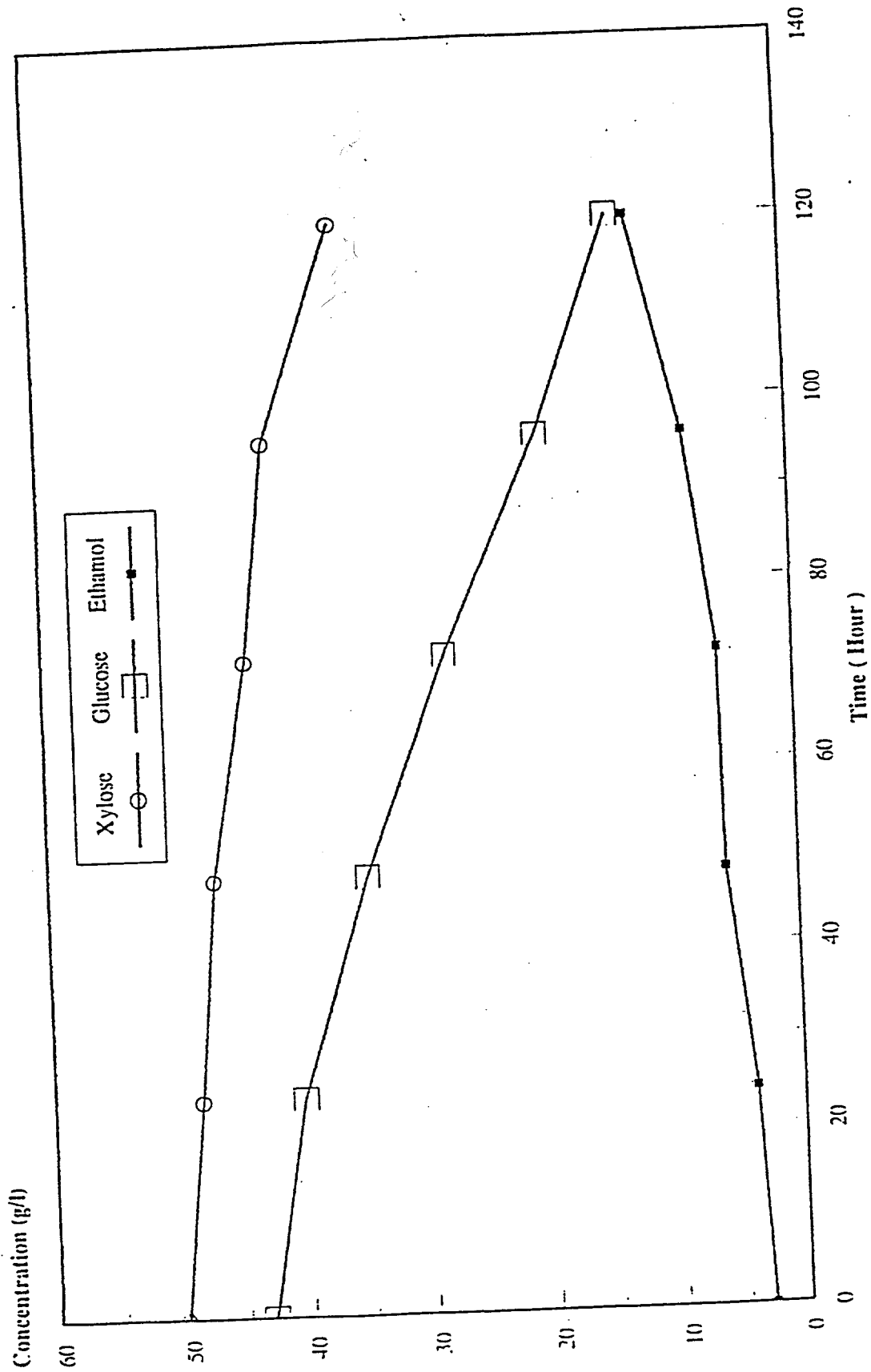


Figure 10. Fermentation of 4% Glucose/5% D-Xylose with *Pichia stipitus* 7124

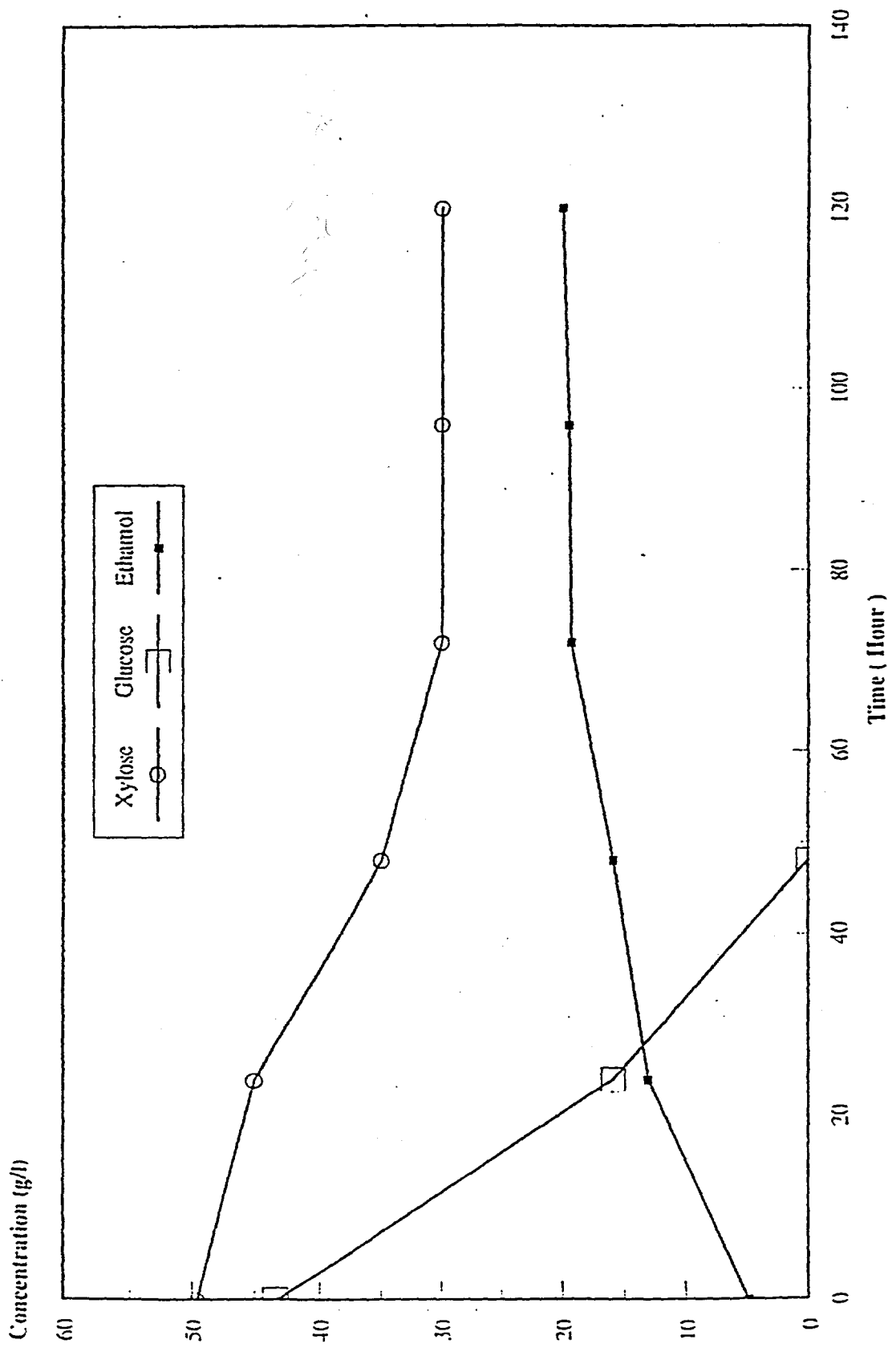
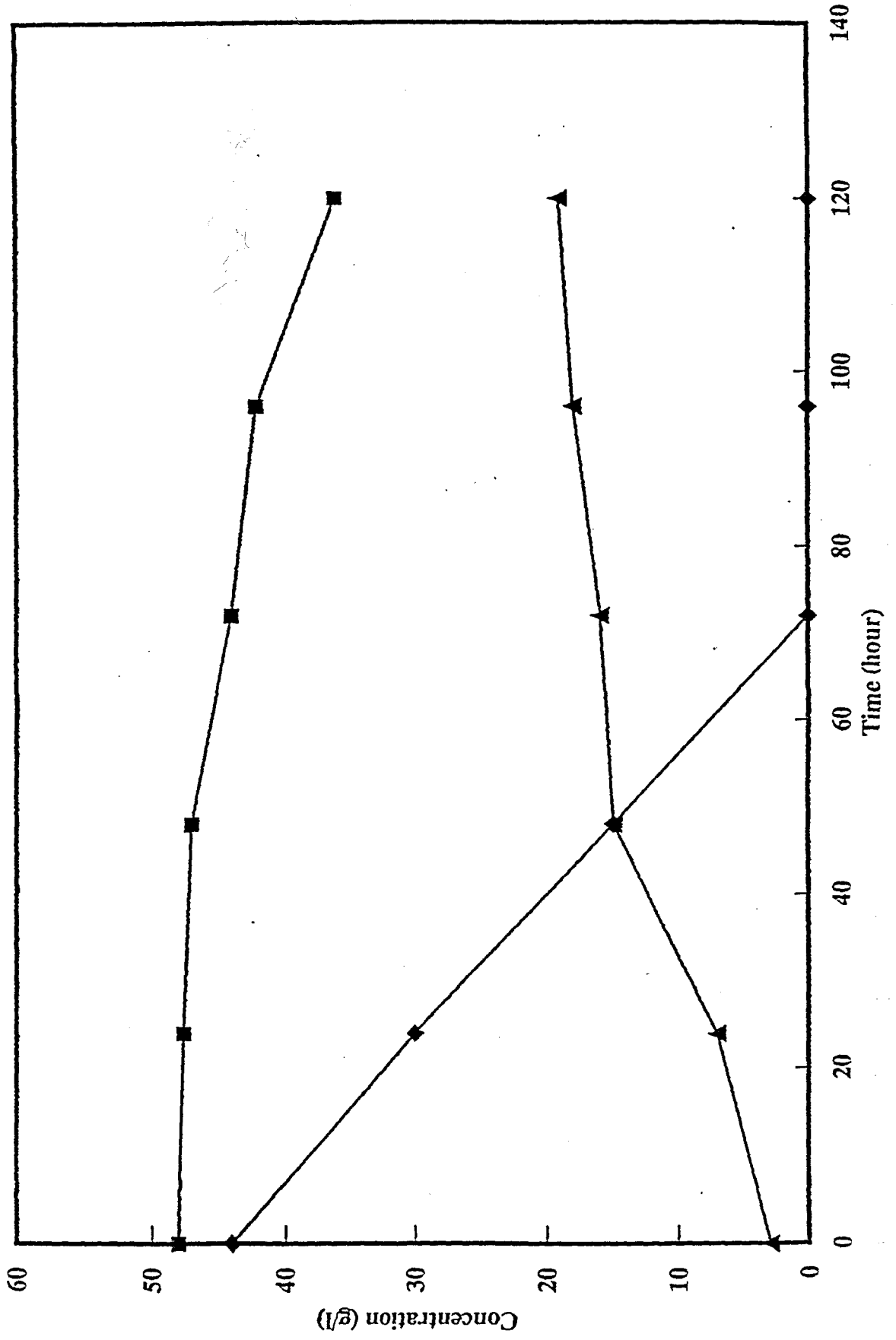


Figure 11. Fermentation of 4% Glucose/5% D-Xylose with *Pachysolen tannophilus* 2460

Figure 12. Fermentation of 4% Glucose/5% D-Xylose with *Pichia segobiensis* 11751



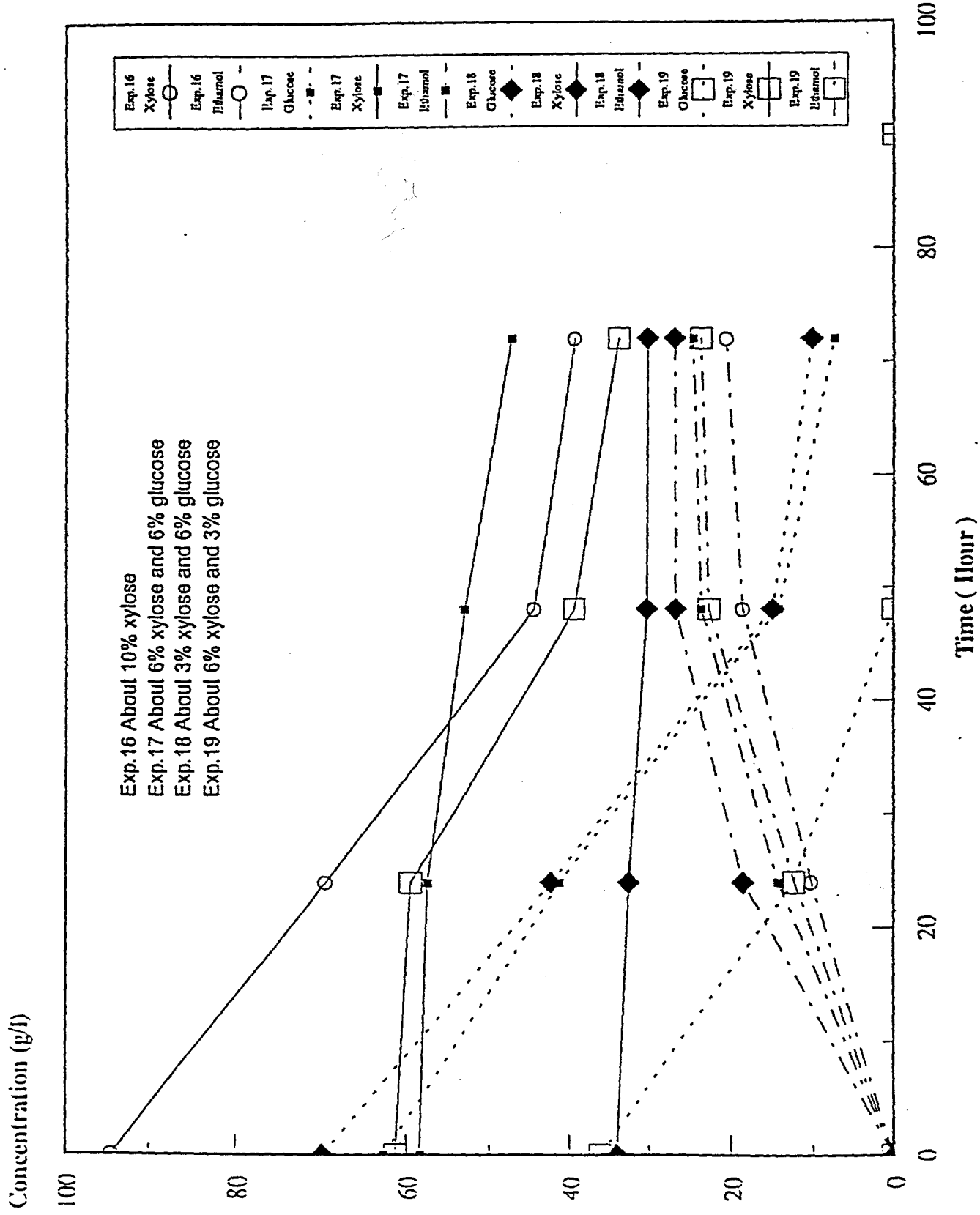
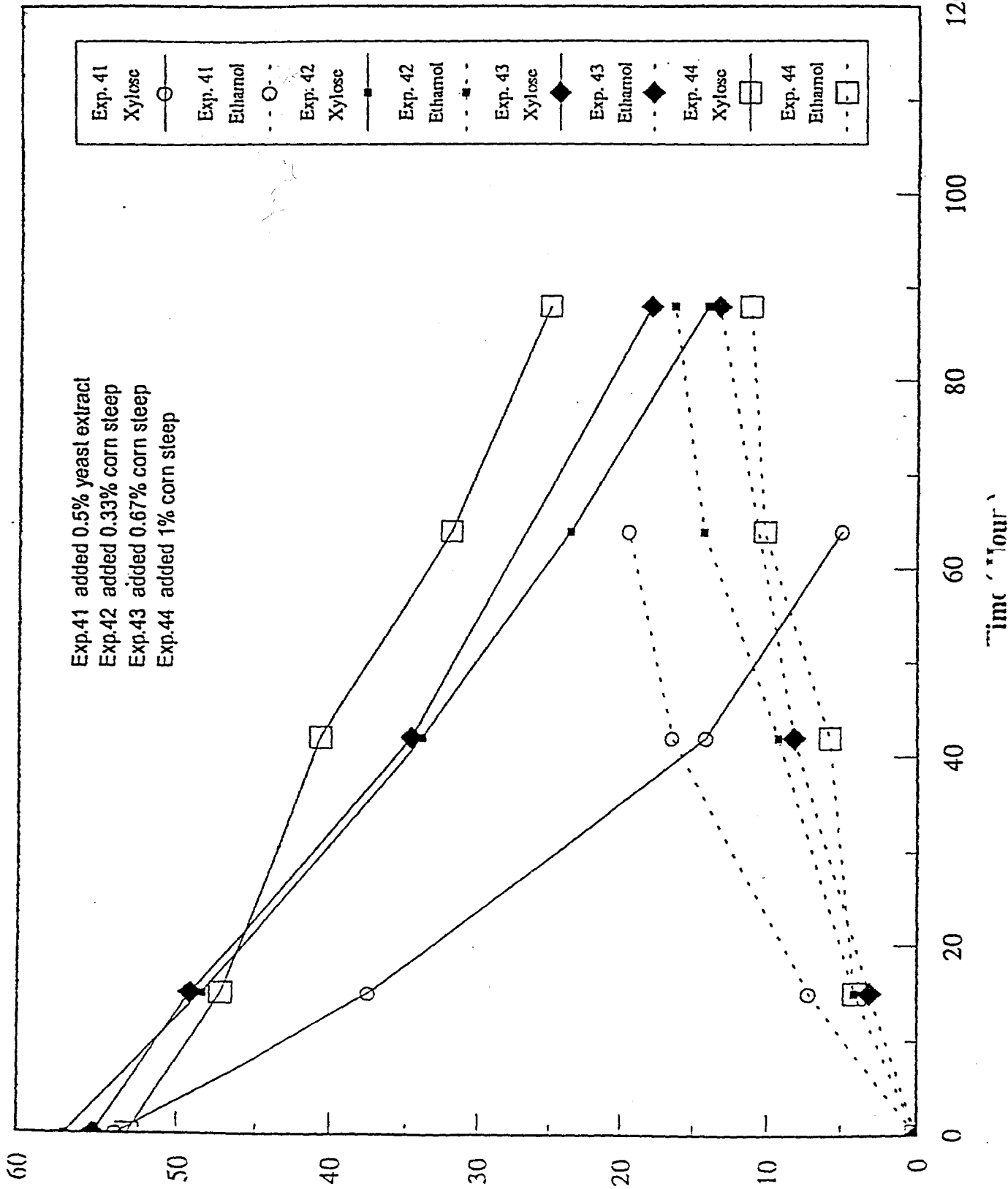


Figure 13. Fermentation of Glucose/ D-Xylose with *Pichia stipitus* 11545 (11545)

Concentration (g/l)



(11545)