SUMMARY Starch-containing plastic films exposed to a natural freshwater environment were shown previously to undergo significant depletion of the starch components. The culture media from a number of starch-hydrolyzing bacteria that had been collected from larvae attached to these films were found to have α-amylase activity. Levels of amylase activity increased with culture age. Most of the activity was found to be cell-associated, and correlated on starch zymograms with an activity at about 55 kDa, in the >50% ammonium sulfate fractionation sample. The pH optimum for these amylases was just at or slightly above neutral, with a temperature optimum of about 65°C.

INTRODUCTION

Many factors contribute to the degradation of composite materials in the environment. Some of the more promising composites are starch-containing plastics (Otey and Doane, 1987). Physical, mechanical and chemical factors all play important roles in breaking these down. Thin films of these materials may be ingested by insect larvae (Imam et al., 1992 Imam et al., 1991) and/or attacked by microbial enzymes which break down their starch components (Burgess-Cassler et al., 1991a; Burgess-Cassler et al., 1991b; Gould et al., 1990).

Very little is known about the in situ roles microbial amylases might play in the degradation of starch-plastics in aqueous environments. The fact that such enzymes do occur in bacteria associated (via pond larvae) with starch-plastics placed in the environment suggests that they may be involved in the breakdown of these materials. Amylase activities described here were initially discovered in the laboratory in such bacterial growth media; it was subsequently established that they are primarily cell-associated, since much higher levels of total amylase activities could be isolated from the bacterial cells.

MATERIALS AND METHODS

Cultivation of microbes. Previously, eleven bacterial strains (designated Mk-039 through Mk-049), were isolated from midge-fly larvae and characterized (Imam et al., 1992). These were grown in trypticase soy broth in stationary tube cultures (~10 ml volumes) at 28-30°C. To obtain sufficient material for more in-depth analyses, washed cell pellets from similar or larger-volume cultures were sonicated for 4-5 min; debris was removed by centrifugation at ≥140,000 x g.

Isolation and electrophoresis of amylase. Amylase activities in culture fluids were measured directly, following clarification by centrifugation and ultrafiltration. Enzyme corresponding to the amylase activities from sonicated cells was obtained from cell pellets (see above) and tested directly, or after ammonium sulfate fractionation (0-50% and 50-100% saturation), followed by extensive diafiltration (Amicon, 10,000 cut-off membrane) of the precipitated protein against HEPES buffer (50 mM HEPES,
pH 7.5, 50 mM NaCl, 2 mM CaCl₂, 5 mM sodium azide). SDS-PAGE was carried out according to the method of Laemmli (1970) and silver staining according to Giulian et al. (1983).

**Amylase assays.** Three different methods were used to assess amylolytic activity. In the first, small aliquots (typically 10 or 20 μl) were used in a standard microplate assay, described previously (Burgess-Cassler and Imam, 1991). This method used a clinical α-amylase determination kit (DMA, Inc., Arlington, TX) with volumes adapted to a microplate format. By definition, 1 U of amylase activity liberates 1.0 μmol of p-nitrophenol/L/min from the maltodextrin-based substrate.

Secondly, since the kit could not be used to determine activity under different pH or temperature conditions, an iodine-uptake/maltodextrin digestion assay, also done in microplate format, was used to determine relative activities in these cases (Burgess-Cassler and Imam, 1991). Finally, amylolytic activity following SDS-PAGE was monitored in situ using starch-zymography, also as described previously (Burgess-Cassler and Imam, 1991; Burgess-Cassler et al., 1991a). This involves renaturing the enzyme at neutral pH within the gel following electrophoresis, then impregnating the gel with a pH 7.50 mM MOPS/0.5% (w/v) gelatinized starch solution. Developing in an I₂/KI solution gives a brown background with cleared zones corresponding to the regions of starch-depletion.

**RESULTS**

Detection of strains with extracellular amylolytic activity. Of the eleven distinct bacterial isolates obtained from midge-fly larvae, five were positive for starch hydrolysis; Mk-039, -040, -041, -042, and -043 (Imam et al., 1992). Cell-free media from all eleven original strains were tested for secreted α-amylase activity versus growth time, using the amylase test kit. While most did not show any detectable such activity, Mk-040, -041, and -042 (referred to here as B, C, and D, respectively) tested positive (Fig. 1).

**Figure 1.**

Microbes B, C, and D (circles, squares, and triangles, respectively) were grown in trypticase soy broth. Samples (10 or 20 μl) were checked for α-amylase activity (clinical kit) as they grew. Only these three strains showed any measurable activity over the time course of the experiment.

The three strains showed similar levels of accumulated α-amylase activity with time, although strain C showed a slightly delayed response. Efforts to obtain larger amounts of amylase by stirred-cell concentration of cell-free spent culture medium were not very successful. The cell pellets from strains B-D were next tested for the presence of amylase, following sonication (using light microscopy, an estimated 50% or so of the cells were broken in this manner). Amylase from a four-day old culture, based on figure 1, would amount to about 3-8 units total. Total amounts from similar volumes of cell-free lysate, based on a 50% yield, were on the order of 30-40 units (some five- to ten-fold higher).

**Optimum pH and Temperature for amylase in cell-free lysates.** Amylase associated with cell-free lysates was tested using the maltodextrin digestion assay to determine optimum pH (Fig. 2). The curves were very similar for the three lysates, with a pH optimum at or just slightly above 7.0. The temperature optima for the zymogram-positive 30-100% saturated fraction revealed that the activity in each case fell